REMARKS

Claims Status

Claims 1 and 3-30 are pending in the subject application.

Response to Restriction Requirement

The Examiner acknowledges Applicants' response to the prior restriction requirement filed on April 15, 2008 and states that the prior restriction is vacated.

Lack of Unity

The Examiner continues to allege that the subject application contains inventions or groups of inventions that are not so linked as to form a single general inventive concept under PCT Rule 13.1. The Examiner now requires restriction and election of a single invention from the Examiner's following Groups 1 to 4:

Group 1, claims 1, 3-21, and 26-30 drawn to a composition comprising an SSRI and a $GABA_B$ receptor antagonist and a method of treating a disorder responsive to SSRIs.
Group 2, claims 1, 3-21, and 26-30 drawn to a composition comprising an SSRI and a $GABA_B$ receptor inverse agonist and a method of treating a disorder responsive to SSRIs.
Group 3, claims 1, 3-21, and 26-30 drawn to a composition comprising an SSRI and a $GABA_B$ receptor partial agonist and a method of treating a disorder responsive to SSRIs.
Group 4, claims 22-24 as it relates to a method of treating of identifying compounds useful in treating depression.

Group 5, claim 25 as it relates to a compound identified by the method of Group 4.
Applicants point out that although the Examiner refers in the Office Action to claims drawn to a composition comprising an SSRI, the independent claims of the invention are drawn to a composition comprising an SRI.

The Examiner states that under the PCT's unity of invention, special technical features are defined as technical features that identify a contribution which each of the claimed inventions,

considered as a whole, makes over the prior art. The Examiner alleges that the first stated technical feature is use of a composition for treating a disorder, and that Group I includes the first composition, and the corresponding method of treatment. The Examiner further alleges that the remaining groups are drawn to different methods of using structurally and functionally diverse compositions. The Examiner further alleges the product groups are drawn to distinct products that lack a special technical feature in common.

The Examiner further alleges that the application contains claims directed to more than one species of the generic invention. The Examiner further alleges that the species are deemed to lack unity of invention because they are not so linked as to form a single general inventive concept under PCT rule 13.1. The Examiner lists the disorders recited in claims 1, 4, 5, 22, 26 and 28.

In response to this Restriction Requirement, Applicants elect, with traverse, the invention of Group 1, i.e., claims 1, 3-21, and 26-30, drawn to a composition comprising an SRI and a $GABA_B$ receptor antagonist and a method of treating a disorder responsive to SRIs. Applicants further elect, with traverse, depression as the species.

Applicants traverse the Examiner's allegation of lack of unity under PCT Rule 13.1 because the inventions of Groups 1-5 are unified by the technical feature of a combination of a $GABA_B$ receptor antagonist, inverse agonist or partial agonist and a SRI. Applicants maintain that the combination of a $GABA_B$ compound and an SRI compound are not disclosed in the prior art, and therefore the technical relationship among the claims as a whole is Applicants' contribution over the prior art.

Therefore unity of invention in accordance with PCT Rules is proper, and Applicants respectfully request that the restriction be withdrawn. To this end:

International Preliminary Examination Report (IPER)

First, the Examiner is respectfully reminded that the decision with respect to unity of invention rests with the International Searching Authority or the International Preliminary Examining

Authority (*see*, e.g., §10.05 of Chapter 10, Unity of Invention, PCT Search and Preliminary Examination Guidelines (2004), p. 75.)

According to the May 28, 2004 IPER, a copy of which was submitted accompanying Applicants' previous response filed April 15, 2008, the International Preliminary Examining Authority did **not** find a lack of unity of invention for PCT/DK03/00412, the International Application on which the present [national stage] application is based. The International Preliminary Examining Authority's decision, therefore, further supports Applicants' position that the restriction of Groups 1-5 is improper.

Technical features

Second, Applicants respectfully point out that PCT Rule 13.1 recites in part: "The international application shall relate to one invention only or to *a group of inventions* so linked as to form a single general inventive concept ...." (Emphasis added.)

Further, PCT Rule 13.2 explains:

Where a group of inventions is claimed in one and the same international application, the requirement of unity of invention referred to in Rule 13.1 shall be fulfilled only when there is a technical relationship among those inventions involving one or more of the same or corresponding special technical features. The expression "special technical features" shall mean those technical features that define a contribution which each of the claimed inventions, *considered as a whole*, makes over the prior art.

Applicants maintain that the claims of the subject application are unified by the technical feature of a combination of a $GABA_B$ receptor antagonist, inverse agonist or partial agonist and a SRI. Applicants maintain that the technical relationship among the claims *as a whole* is Applicants' contribution over the prior art.

On page 2 of the Office Action, the Examiner acknowledges that the Examiner's failure to provide a prior art teaching of the claimed compositions and methods in the prior restriction was

sufficient basis so as to vacate it. Similarly, since the Examiner has not established that the special technical feature of the present application is known (e.g., by providing a prior art teaching), the technical feature is a contribution over the prior art, and thus, the present invention has unity.

Markush Alternatives

Third, Applicants also point out PCT Rule 13.3, which recites:

The determination whether a group of inventions is so linked as to form a single general inventive concept shall be made *without* regard to whether the inventions are claimed in separate claims or as *alternatives within a single claim.* (Emphasis added.)

The Examiner has restricted claims 1, 3-21, and 26-30 based on the $GABA_B$ alternatives, i.e. a $GABA_B$ receptor antagonist, inverse agonist, *or* partial agonist. According to PCT Rule 13.3, the Examiner's restriction of Groups 1-3 is improper because the Examiner has focused Groups 1-3 on the alternatives within a claim.

Guidance for determining unity of invention is given in MPEP §1850 (III)(B), which states "the requirement of a technical interrelationship and the same or corresponding special technical features as defined in PCT Rule 13.2, shall be considered to be met when the alternatives are of a similar nature."

MPEP §1850 (III)(B) further recites "[w]hen the Markush grouping is for alternatives of chemical compounds, they shall be regarded as being of a similar nature where the following criteria are fulfilled:

(A) *All alternatives have a common property or activity; and*

(B) (1) A common structure is present, i.e., a significant structural element is shared by all of the alternatives; *or*

(B) (2) In cases where the common structure cannot be the unifying criteria, *all alternatives belong to a recognized class of chemical compounds* in the art to which the invention pertains." (Emphasis added.)

Applicants maintain that "$GABA_B$ receptor antagonist, inverse agonist, or partial agonist" is an art-recognized term to describe the class of $GABA_B$ receptor-modulating compounds. Prior to the filing of the subject application, it was known that an antagonist may be classified as inverse agonist or partial agonist based on the assay used to determine receptor activity. *See*, e.g. de Ligt, R.A.F. et al., *British Journal of Pharmacology*, 2000, 130:1-12; Kenakin, T., *FASEB Journal*, 2001, 15:598-611; and Strange, P.G., *Trends in Pharm. Sciences (TiPS)*, 2002, 23(2):89-95 (copies of which are attached).

For example, de Ligt et al. teaches that the classification of ligands depends on the specificity of the assay, and that "...the same ligand may behave as an inverse agonist, a neutral antagonist or even a partial agonist". (*See* page 2, column 1, paragraph 3, last line; Emphasis added.) Kenakin defines the art-recognized meaning of partial agonist, antagonist and inverse agonist on page 599, Figure 1, and explains that based on the current knowledge as of 2001 "...most antagonists are inverse agonists" (page 602, column 1, last paragraph). Kenakin also explains on page 602, column 1, paragraph 2, that new receptor assay systems were emerging, and "...now that more laboratories have the eyes to see inverse agonism, the more it has been seen."

Applicants maintain one skilled in the art would recognize that a compound characterized as a $GABA_B$ antagonist, $GABA_B$ inverse agonist or $GABA_B$ inverse agonist, belongs to a recognized class of chemical compounds of which the latter are alternatives. Accordingly, the Examiner's groups are linked as to form a single general inventive concept, and the present invention has unity.

Conclusion

For the foregoing reasons, Applicants' traverse the Examiner's requirement for restriction under the PCT Rules and respectfully request that the restriction be withdrawn.

If a telephone interview would be of assistance in advancing prosecution of the above-identified application, Applicant's invite the Examiner to telephone the undersigned at the number provided below.

Authorization is hereby given to charge any additional fee(s), or credit any overpayment, to Deposit Account No. 50-3201.

Respectfully submitted,

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British Journal of Pharmacology (2000) 130, 1–12
www.nature.com/bjp

REVIEW

Inverse agonism at G protein-coupled receptors: (patho)physiological relevance and implications for drug discovery

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British Journal of Pharmacology (2000) **130,** 1–12

Keywords: GPCR; inverse agonism; neutral antagonist; negative efficacy; review; implications drug discovery

Abbreviations: Ado, adenosine; AM630, 6-iodopravadoline; AR, adrenoceptor; BK, bradykinin; CAM, constitutively active mutant; CB, cannabinoid; CHO, Chinese hamster ovary; COS, African green monkey kidney fibroblast-like cell line; CP-55940, (−)-3-[2-hydroxy-4-(1,1-dimethylhyptyl)phenyl]-4(3-hydroxypropyl)cyclohexan-1-ol; CT, calcitonin; 5-CT, 5-carboxamidotryptamine; D, dopamine; DMCM, methyl 6,7-dimethoxy-4-ethyl-β-carboline-e-carboxylate; DOI, (±)-1-(2,5-dimethoxy-4-iodophenyl)-2-aminopropane; DPCPX, 1,3-DiPropyl-8-cyclopentyladenosine; F314, [Mpa, D-Tyr(Ethyl),Thr, Orn]-oxytocin; FMLP, *N*-formyl-L-methionyl-L-leucyl-L-phenylalanine; FP, formyl peptide; FSH, follicle-stimulating hormone; GLP-1, glucagon-like peptide-1; GPCR, G protein-coupled receptor; GTP, guanosine-5′-triPhosphate; GTPase, guanosine-5′-triphosphatase; [^{35}S]-GTPγS,-[^{35}S] guanosine 5′-*O*-(thiotriphosphate); H, histamine; HEK293, human embryonic kidney; H.E.L. 92.1.17, human erythroleukemia; HL-60, human leukemia; I_{Ca}, L-type calcium currents; ICI-118,551, (±)-1-(2,3-[dihydro-7-methyl-1*H*-inden-4-yl]oxy)-3-([1-methylethyl]-amino)-2-butanol; ICI-174,864, *N-N*-diallyl-Tyr-Aib-Aib-Phe-Thr; IP, inositol phosphates; IP-10, interferon-γ-inducible protein 10; LH, luteinizing hormone; M, muscarinic; MAPK, mitogen-activated protein kinase; MC, melanocortin; Mig, monokine induced by interferon-γ; α-MSH, melanocyte stimulating hormone; NG108-15, neuroblastoma X glioma hybrid; O, opioid; P, purinergic; PTH, parathyroid hormone; PTHrP, parathyroid hormone-related peptide; QNB, *R*(−)-quinuclidinylbenzilate; RIN5AH, rat insulinoma-derived; Sf9, *Spodoptera frugiperda* 9; SB206553, *N*-3-pyridinyl-3,5-dihydro-5-methylbenzo[1,2-b:4,5-b′]dipyrrole-1(2H)-carboxamide hydrochloride; SDF-1α, stromal cell-derived factor 1α; SR141,716A, *N*-(piperidino-1-yl)-5-(4-chlorophenyl)-1-(2,4-dichlorophenyl)-4-methyl-pyrazole-3-carboxamide; SR144,528, *N*-([1s]-endo-1,3,3-trimethylbicyclo[2.2.1]heptan-2-yl)-5-(4-chloro-3-methylphenyl)-1-(4-methylbenzyl)-pyrazole-3-carboxamide; vMIP-II, viral macrophage inflammatory protein-II; WAY100,635, N-{2-[4-(2-methoxyphenyl)-1-piperazinyl]ethyl}-N-(2-pyridinyl)-cyclohexane-carboxamide; WIN55,212-2, R(+)-[2,3-dihydro-5-methyl-3-[(morpholinyl)methyl]pyrrolo[1,2,3-*de*]-1,4-benzoxazin-yl]-(1-napthalenyl)methanone mesylate; WB4101, 2-(2,6-dimethoxyphenoxyethyl)aminomethyl-1,4-benzodioxane; XAC, xanthin amino congener

Introduction

The largest family of cell surface receptors involved in signal transduction, G protein coupled receptors (GPCRs), are one of the major targets for current drugs as well as new drug development. Ligands interacting with for e.g. adrenergic, histamine, adenosine, opioid, dopamine or serotonin receptors, constitute a large portion of currently used therapeutics. A common property of GPCRs is that upon activation (agonist binding) they transmit signals across the plasma membrane *via* an interaction with heterotrimeric G proteins (Stadel *et al.*, 1997). The corresponding activated G protein subsequently interacts with an intracellular effector system, such as adenylate cyclase or phospholipase C, leading to a wide variety of distinct physiological responses.

Recent evidence suggests that GPCRs have the potential to be 'active' even in the absence of an agonist. This exhibition of spontaneous receptor activity has led to the observation that various ligands, previously considered as antagonists with no intrinsic activity, actually can inhibit this spontaneous activity, appearing to possess 'negative intrinsic activity'. This phenomenon has been termed inverse agonism and the corresponding ligands are referred to as inverse agonists.

Although intrinsic constitutive receptor activity and inverse agonism have unequivocally been demonstrated *in vitro*, (patho)physiological consequences are far from self-evident. Thus, in this review we should like to focus on the expression of inverse agonism under more 'physiological conditions', since it appears timely to address the physiological relevance and consequences of this new concept, both in GPCR research and drug discovery.

Historical overview

Traditional receptor theory has postulated on a single, 'quiescent' receptor state to which agonists bind inducing a conformational change of the receptor to an activated and 'functional' state. This view, initially formed in the early 1950s, was more clearly expressed by Del Castillo & Katz (1957), and stood as the foundation of receptor pharmacology for decades. It was believed that antagonists interact with the receptor, thereby preventing agonist binding, without having an effect on conformational changes of the receptor that remained in its 'quiescent' state.

The first evidence of a ligand ('antagonist') producing opposite effects to those of an agonist, and thus not merely inhibiting agonist binding, stems from the GABA-benzodiazepine field. In 1982, Braestrup *et al.* reported on the discovery of an agent, DMCM (methyl 6,7-dimethoxy-4-ethyl-β-carboline-e-carboxylate), which in contrast to benzodiazepines, not only was a potent convulsant *in vivo*, but seemed to favour

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binding to benzodiazepine receptors that were in the non-GABA(agonist)-stimulated conformation. For the first time, it was elaborated that GABA-benzodiazepine receptors may perhaps exist in two conformations which are in equilibrium, an open chloride channel form (activated conformation) and a closed one (inactivated conformation) for which DMCM may have a high affinity and a tendency to stabilize, thereby decreasing binding of GABA to the activated conformation.

Interestingly, the above concept of an 'agonist-independent' two-state receptor conformation, introduced initially for ion-channel-coupled receptors, soon found support from those studying the large family of GPCRs. Costa & Herz (1989) were pioneers in setting the grounds for what later would be referred to as 'inverse agonism'. They demonstrated for the first time that some antagonists of the δ opioid receptor had 'negative intrinsic activity' *in vitro*, in contrast to others that lacked any intrinsic activity. Moreover, such ligands diminished even further the 'basal' or constitutive activity of the receptor defined as the activity of the receptor in the absence of any ligand. It was apparent that the concept of 'negative intrinsic activity' implied a pre-existing equilibrium between (at least) two states of a receptor. These two states could easily be defined as either a G protein-bound or a free form of the receptor, the first one active and the latter inactive. In their studies, basal GTPase activity in NG108-15 cell membranes was suggested to be due to stimulated activity resulting from a spontaneous interaction between empty or free receptors and G proteins. Their data lent support to the receptor model of Wregget & De Lean (1984) which predicted that 'antagonists may be *active* by hindering the ability of receptors to associate spontaneously with G proteins in membranes'.

Since then, and especially over the past few years, disclosure of negative intrinsic activity has corroborated even further this two-state model of GPCR activation. Hence, a large number of publications within this decade are concerned with, and demonstrate with a variety of systems or means, the phenomenon of constitutive receptor activity and its implication for inverse agonism.

In this review we will first address some of the more seminal papers, showing that in most cases genetically engineered cell systems were pivotal for the development of this new concept. These and many more studies have been adequately and thoroughly reviewed recently by Milligan *et al.* (1997) and Leurs *et al.* (1998). We will then gradually move to more 'physiological' systems, in order to address the central issue of this review whether inverse agonism and spontaneous receptor activity are relevant phenomena in health and disease, and hence for drug discovery. It should be pointed out, however, that a classification of ligands based on their (negative) intrinsic activity is not an easy task. Due to the large influence of receptor systems and experimental conditions (whole cells versus membranes, stoichiometry of receptors/G protein, signalling proteins etc.) the same ligand may behave as an inverse agonist, a neutral antagonist or even a (partial) agonist.

Genetically engineered systems to facilitate detection of inverse agonism

CAMs and receptor or G protein overexpression Inverse agonism on GPCRs is not always easily established, since basal receptor activity is generally not pronounced. Thus, various manipulations to increase basal (constitutive) receptor activity have been explored, such as construction and expression of constitutively active mutant receptors (CAM) or overexpression of either the receptor or the G protein to favourably change the receptor-G protein ratio (R : G).

CAM receptors show higher agonist-independent activity and have been reported for various receptor subtypes. Since CAM receptors have a higher basal receptor activity, the effect of inverse agonists is more readily observed. Samama *et al.* (1993) were the first to describe a CAM receptor of the β_2-adrenoceptor (AR); replacement of four amino acids of the third intracellular loop by the corresponding residues of the α_{1B}-AR, led to agonist-independent activation of adenylate cyclase. Previous work by Cotecchia *et al.* (1990) had demonstrated that substitution of residues in the third intracellular loop of the β_2-AR by the corresponding residues of the α_1-AR, led to chimeric receptors that were coupled to PI hydrolysis, like the native α_1-AR, instead of adenylate cyclase. This provided direct evidence that the third intracellular loop is important for G protein binding and activation.

Since normal expression levels of wild type receptors do not always result in constitutive activity, numerous studies have been performed in various systems where overexpression of the wild type receptor has been induced. Examples of these are the expression of wild type β_2-AR in Sf9 insect cells leading to receptor densities up to 40 pmol mg^{-1} protein (Chidiac *et al.*, 1994) or the overexpression of the calcitonin receptor in HEK293 cells (Pozvek *et al.*, 1997). In the latter case, two different clonal cell lines were selected, expressing 5×10^6 and 25×10^3 receptors/cell, respectively. Whereas the first cell line displayed an 80 fold increase in basal cyclic AMP production, the second was not constitutively active. Apparently, receptor density is positively correlated to spontaneous activity and various classes of GPCRs can display constitutive activity upon overexpression.

Overexpression of the G protein involved may also lead to increased basal levels of second messengers. High levels of $G_{\alpha q}$ cotransfected with various muscarinic receptor subtypes in NIH3T3 cells resulted in increased basal activity of the receptors (Burstein *et al.*, 1997). This induced constitutive activity of the receptors was reversed completely by the muscarinic antagonists tested, indicating that they behaved as inverse agonists. Thus, elevation of G protein levels favours formation of the active conformation of the receptor the fraction of receptors that are coupled to the G protein and provides a more sensitive means for the detection of inverse agonism.

These genetic approaches even work *in vivo*, since transgenic animals overexpressing GPCRs proved another source of constitutively active receptors. Bond *et al.* (1995) described transgenic mice overexpressing the wild type β_2-AR at various receptor levels. Baseline left atrial tension in these transgenic mice was increased 3 fold over control mice while the β_2-selective ligand ICI-118,551, acting as an inverse agonist, decreased baseline tension. The inhibitory effect of ICI-118,551 was correlated with β_2-AR densities, suggesting that it was a receptor-mediated event. Apart from these organ bath data, the effect of ICI-118,551 was also studied *in vivo*, where cardiac contractility was measured in both control and transgenic mice. ICI-118,551 decreased cardiac contractility in transgenic mice by approximately 70%, an effect that was associated with a fall both in heart rate and left ventricular systolic pressure, while the compound exhibited no effects on control hearts. Recently, the CAM β_2-AR mentioned above, has also been overexpressed in mice (Samama *et al.*, 1997). In this case, the mouse phenotype was not very different from normal, probably due to the rather modest overexpression ($\sim$3 fold). However, long-term treatment with ICI-118,551 increased CAM β_2-AR density, resulting in marked basal atrial tension and cardiac contractility. Finally, Nagaraja *et al.* (1999) described the effects of long term treatment of various β-

adrenoceptor ligands on baseline left atrial tension in transgenic mice with modest β_2-AR overexpression (50 fold compared to 200 fold β_2-AR overexpression in the paper by Bond *et al.* (1995)). In these transgenic mice, inverse agonists such as ICI-115,881, carvedilol and propranolol increased baseline left atrial tension, whereas untreated or alprenolol-treated mice were unaffected. Baseline left atrial tension was not affected by any ligand in the hearts of wild type mice. This paper therefore showed the differential effects of a neutral (alprenolol) and inverse agonists (e.g. ICI-118,551) besides the importance of receptor density in the study of inverse agonism.

Thus, constitutive receptor activity has been demonstrated for several GPCRs after some form of genetic manipulation. We will now review other types of studies that may have a more direct link to *in vivo* pharmacology and physiology. These studies include cell systems with 'normal' levels of receptor expression and tissue or organ bath preparations. The issue of 'endogenous' inverse agonists will also be discussed.

Inverse agonism at wild type receptors Various authors have described inverse agonism and constitutive activity on wild type receptors expressed in artificial cell lines but at more or less 'physiological' levels of expression. Examples of such studies are listed in Table 1 and discussed below.

The rat histamine H_2 receptor stably transfected in CHO cells was studied by Smit *et al.* (1996). This receptor had pronounced basal activity, as indicated by an increase in basal cyclic AMP production. Although this basal cyclic AMP was further increased by the endogenous agonist histamine, the H_2 blockers cimetidine and ranitidine were shown to decrease basal cyclic AMP production, therefore expressing inverse agonism in this system. Burimamide, on the other hand, did not alter basal cyclic AMP levels but was able to block both the histamine-induced increase and the cimetidine-induced decrease of basal cyclic AMP production. Hence, burimamide behaved as a neutral antagonist. Similar results were obtained for the human histamine H_2 receptor (Alewijnse *et al.*, 1998), although at this receptor burimamide behaved as a weak partial agonist, increasing basal cyclic AMP production by 16%, compared to the maximal response induced by histamine.

Newman-Tancredi *et al.* (1997) studied the 5-HT_{1A} subtype of the serotonin receptor expressed in CHO cells at a receptor density of 1.6 pmol mg^{-1} protein. Modulation of [^{35}S]-GTPγS binding in a membrane preparation was used to discriminate between the various ligands tested. 5-Carboxamidotryptamine (5-CT) was classified as a full agonist increasing [^{35}S]-GTPγS binding to the same extent as serotonin (5-HT), whereas spiperone was identified as an inverse agonist since it decreased basal [^{35}S]-GTPγS binding by 30%. Meanwhile, WAY100,635 behaved as a neutral antagonist. It showed no effect on basal [^{35}S]-GTPγS binding by itself, but was able to block both 5-CT-induced stimulation and spiperone-induced inhibition of basal [^{35}S]-GTPγS binding. The effect of spiperone could not be explained by a simple displacement of endogenous 5-HT; not only had the membranes been extensively washed, but if basal activity had been due to 5-HT_{1A} receptor activation by endogenous 5-HT, the antagonist WAY100,635 should have also blocked this activation.

The behaviour of both subtypes of the human cannabinoid (CB_1 and CB_2) receptor was analysed by Bouaboula *et al.* (1997; 1999). CHO cells, stably expressing either the CB_1 or the CB_2 receptor, showed higher basal MAPK activity compared to untransfected CHO cells. In both transfected cell lines CP-55940, a non-selective cannabinoid agonist, further increased basal MAPK activity. The CB_1-selective compound SR141,716A decreased basal MAPK activity in CHO cells expressing the CB_1 receptor, thus behaving as an inverse agonist for this receptor (Bouaboula *et al.*, 1997). SR141,716A also displayed effects opposite to agonists in a cyclic AMP-related luciferase assay. Instead of a decrease in luciferase activity induced by cannabinoid agonists, SR141,716A elicited an increase. Apparently, SR141,716A acted as an inverse agonist in two different signal transduction pathways, i.e. $G_{\beta\gamma}$-mediated MAPK-activity and $G_{\alpha,i}$-mediated adenylate cyclase inhibition. Similar results were obtained with SR144,528, a CB_2-selective ligand that behaved as an inverse agonist, decreasing both basal MAPK activity and [^{35}S]-GTPγS binding on CHO cells expressing the CB_2 receptor (Bouaboula *et al.*, 1999). Furthermore, Landsman *et al.* (1998) reported on another inverse agonist for the human CB_1 receptor, AM630, which decreased basal [^{35}S]-GTPγS binding, in contrast to the cannabinoid agonist WIN55,212-2.

Inverse agonistic effects were also shown at various dopamine receptor subtypes. Tiberi & Caron (1994) reported basal receptor activity of the dopamine D_{1A} and D_{1B} receptor, the human D_{1B} receptor being linked to higher intracellular basal cyclic AMP levels, compared to the D_{1A} receptor. Similar results were obtained for the corresponding rat receptors. Two dopamine antagonists, (+)-butaclamol and flupentixol, were able to decrease basal cyclic AMP levels; the inhibitory effect of these compounds, acting as inverse agonists, was more pronounced at the human D_{1B} receptor, since the basal cyclic AMP level was higher. More recently, Griffon *et al.* (1996) showed that various antipsychotics inhibited [^{3}H]-thymidine incorporation in NG108-15 cells expressing the recombinant human dopamine D_3 receptor. Since dopamine agonists enhanced [^{3}H]-thymidine incorporation, the antipsychotics tested (haloperidol, fluphenazine and chlorpromazine), behaved as inverse agonists. Nafadotride, a D_3 receptor-preferring antagonist, had no effect of its own on [^{3}H]-thymidine incorporation, therefore behaving as a neutral antagonist.

Table 1 Examples of constitutively active wild type receptors expressed at more or less 'physiological' receptor levels

Receptor	*Cell line*	B_{max} (pmol mg^{-1} protein)	*Reference*
Rat dopamine D_{1A} and D_{1B}	HEK293	2	Tiberi & Caron, 1994
Rat histamine H_2	CHO	Variable (0.3–1.0)	Smit *et al.*, 1996
Human dopamine D_3	NG108-15/CHO	n.d.	Griffon *et al.*, 1996
Human calcitonin CTR-1 or -2	COS-1	Variable	Cohen *et al.*, 1997
Human serotonin 5-HT_{1A}	CHO	1.6	Newman-Tancredi *et al.*, 1997
Human cannabinoid CB_1	CHO	n.d.	Bouaboula *et al.*, 1997
Human cannabinoid CB_1	CHO	3.2	Landsman *et al.*, 1998
Human formyl peptide FP	Sf9/HEK293	1.1/1.2	Wenzel-Seifert *et al.*, 1998
Human histamine H_2	CHO	1	Alewijnse *et al.*, 1998
Human cannabinoid CB_2	CHO	n.d	Bouaboula *et al.*, 1999

The two isoforms of the human calcitonin receptor (referred to as hCTR-1 and hCTR-2) activate adenylate cyclase, while in addition one of them (hCTR-2) is also able to activate phospholipase C to generate inositol phosphates (IP). Cohen *et al.* (1997) described constitutive receptor activity of human calcitonin receptors. Both hCTR-1 and hCTR-2 receptors expressed in COS-1 cells were constitutively active as shown by an increase in basal cyclic AMP production, although to a different extent. However, the hCTR-2 receptor did not show an increase in basal IP production. Apparently, spontaneous activity of this receptor was more readily observed for activation of adenylate cyclase. Addition of salmon calcitonin (sCT), a calcitonin receptor agonist, increased cyclic AMP production further, whereas the analogue N^{α}-acetyl-sCT-(8-32)amide did not elicit an effect. The paucity of available ligands prevented a further demonstration of inverse agonism.

A last example is the human formyl peptide (FP) receptor, a chemoattractant GPCR, studied by Wenzel-Seifert *et al.* (1998). This receptor was expressed in Sf9 and HEK293 cells at receptor densities of approximately 1 pmol mg^{-1} protein. These receptor levels are in the same range as the receptor density in HL-60 cells that endogenously express the FP receptor. It was shown that basal [^{35}S]-GTPγS binding increased in the presence of *N*-formyl-L-methionyl-L-leucyl-L-phenylalanine (FMLP), a FP receptor agonist. On the contrary cyclosporin H decreased basal [^{35}S]-GTPγS binding, thereby behaving as an inverse agonist. It seems, therefore, that at physiological receptor levels and expressed in different cell lines, the human FP receptor is constitutively active.

Inverse agonism in 'physiological' (or non-engineered) systems

A prerequisite for considering the physiological relevance of inverse agonism, is its study in experimental conditions that are as close to physiological as possible. Data related to inverse agonism obtained in intact animals (*in vivo*) are in most cases difficult to acquire, unless animals are genetically altered (e.g. transgenic mice) as described previously. Thus, apart from potential '*in vivo*' data, other more or less 'physiological studies' may include data from cell lines endogenously expressing the receptor of interest, as for example the previously mentioned NG108-15 cells expressing δ opioid receptors (Costa & Herz 1989), or from tissue preparations such as cardiac or brain cortex membranes. Both latter methods may provide *ex vivo* data of wild type receptors at physiological or pathophysiological levels. Various examples of such studies in which potential inverse agonism was detected are summarized in Table 2.

Hilf & Jakobs (1992) described a decrease in G protein activation by antagonists of the muscarinic receptor in porcine atrial membranes. With this membrane preparation, enriched by sucrose density gradient centrifugation to contain ~1.4 pmol receptors per mg protein, inhibition of both basal and carbachol-induced [^{35}S]-GTPγS binding by atropine was shown. The presence of endogenous acetylcholine (ACh) was ruled out in this study by pretreatment of the membranes with 10 μM atropine or 100 μM GDP and subsequent washes, both compounds displacing all ACh possibly present. Jakubik *et al.* (1995) showed inverse agonism in rat cardiomyocytes expressing the M_2 receptor. Atropine and QNB, both muscarinic antagonists, increased basal as well as forskolin-induced cyclic AMP production, an effect that was opposite to that of agonists. Similar results were obtained for three other subtypes of the muscarinic receptors, M_1, M_3 and M_4, albeit in a 'non-physiological' setting, i.e. in CHO cells stably transfected with the human receptor gene.

To detect inverse agonism in H.E.L. 92.1.7 cells endogenously expressing the human α_{2A}-AR, Jansson *et al.* (1998) used two different assays. Effects of various α_2-adrenoceptor ligands on both intracellular levels of Ca^{2+} ($[Ca^{2+}]_i$) and forskolin-stimulated cyclic AMP production were investigated. The ligands used were thus classified from full agonists to inverse agonists. Both assays gave similar indications of intrinsic activities, showing that different assays and/or signalling pathways can sometimes be used to detect and classify ligands as inverse agonists. Quite remarkable in these studies were the opposite effects of the enantiomers of medetomidine; while dexmedetomidine acted as a partial agonist, increasing $[Ca^{2+}]_i$ and decreasing forskolin-stimulated cyclic AMP production, levomedetomidine behaved as an inverse agonist by decreasing $[Ca^{2+}]_i$ and increasing cyclic AMP production.

Other adrenoceptor subtypes have also been studied to observe inverse agonism in such 'physiological studies'. The α_{2D}-AR, endogenously expressed in RIN5AH cells and heterologously expressed in PC-12 cells, revealed inverse agonistic effects of rauwolscine in [^{35}S]-GTPγS binding assays (Tian *et al.*, 1994). Accordingly, isoprenaline increased GTPγS-induced adenylate cyclase activity *via* the β-AR expressed in turkey erythrocytes, while both propranolol and pindolol showed a decrease, thus behaving as inverse agonists (Götze & Jakobs 1994).

A single cell preparation from cardiac tissue was used in the following two examples. Mewes *et al.* (1993) recorded L-type calcium currents (I_{Ca}) on both guinea-pig and human ventricular myocytes as a means to study the effects of the β-AR antagonists atenolol and propranolol. The myocytes were first superfused with 0.5 μM forskolin, to make the cells more sensitive to receptor-mediated changes of I_{Ca}. Application of both antagonists led to a decrease in forskolin-stimulated I_{Ca} in all myocyte preparations. Successive exposures to atenolol, a hydrophilic compound that could be readily washed away,

Table 2 Inverse agonism in 'physiological' studies

Cell line/tissue	*Receptor*	*Readout*	*Reference*
NG108-15	δ opioid	GTPase	Costa & Herz, 1989
Porcine atrial membranes (enriched)	mACh	[^{35}S]-GTPγS-binding	Hilf & Jakobs, 1992
Guinea-pig/human cardiac myocytes	β-AR	Calcium current (I_{Ca})	Mewes *et al.*, 1993
Frog/rat cardiac cells	mACh	Calcium current (I_{Ca})	Hanf *et al.*, 1993
Rat/bovine myometrial membranes	Bradykinin BK_2	IP-production	Leeb-Lundberg *et al.*, 1994
Turkey erythrocytes	β-AR	AC-activity	Götze & Jakobs, 1994
Rat RIN5AH	α_{2D}-AR	[^{35}S]-GTPγS-binding	Tian *et al.*, 1994
Rat cardiomyocytes	mACh (M_2)	cyclic AMP	Jakubik *et al.*, 1995
Rat thoracic aorta strips	mACh	↑ resting tone (I_{Ca})	Noguera *et al.*, 1996
Guinea-pig ileum	κ opioid	Contraction (twitch and abstinence response)	Cruz *et al.*, 1996
NG108-15	δ opioid	[^{35}S]-GTPγS-binding	Szekeres & Traynor, 1997
H.E.L. 92.1.17	α_{2A}-AR	↑[Ca^2], cyclic AMP	Jansson *et al.*, 1998

resulted in similar changes in I_{Ca}, suggesting that these inhibitory effects were not due to a potential competition with endogenous agonist but to inverse agonistic activity of these antagonists.

Hanf *et al.* (1993) performed a similar study also using cardiomyocytes, but from two different species, frog and rat. They studied I_{Ca} regulated by muscarinic receptors. To increase basal I_{Ca}, isoprenaline was added during the experiments with frog ventricular cells (Figure 1). Addition of ACh resulted in a decrease of this basal I_{Ca}, whereas addition of atropine had the opposite effect, namely an increase of I_{Ca}. A similar effect of atropine was also noticed in the absence of Ach. This effect was dose-dependent and reversible. Results obtained with rat myocytes did not require the addition of isoprenaline since basal I_{Ca} was already high enough to observe the effects produced by the agonist ACh and the inverse agonist atropine.

Organ bath preparations have also been used to detect inverse agonism (Noguera *et al.*, 1996). Helically cut strips of rat thoracic aorta were prepared and the effects of α_1-adrenoceptor antagonists on the regulation of the resting tone, induced by noradrenaline or Ca^{2+} exposure, were studied. Benoxathian and WB4101 not only inhibited the increase in resting tone induced by noradrenaline, but also blocked the response to noradrenaline in calcium-free medium. An explanation for these results was given within the concept of inverse agonism; it was suggested that both benoxathian and WB4101 acted as inverse agonists, decreasing the proportion of the receptor population in the active state (R*). Moreover, both compounds inhibited the increase in resting tone in the absence of agonist, providing a simple model for analysing inverse agonism in functional studies.

Other receptor types showing inverse agonism under 'physiological conditions' are the bradykinin BK_2 receptor and the δ opioid receptor. Briefly, different bradykinin antagonists decreased basal IP production in rat myometrial cells endogenously expressing the BK_2 receptor, while increasing concentrations of bradykinin increased basal IP production (Leeb-Lundberg *et al.*, 1994). Furthermore, ICI-174,864 was shown to behave as an inverse agonist at the δ opioid receptor, endogenously expressed in NG108-15 cells, in both GTPase activity measurements and [^{35}S]-GTPγS binding (Costa & Herz, 1989; Szekeres & Traynor, 1997).

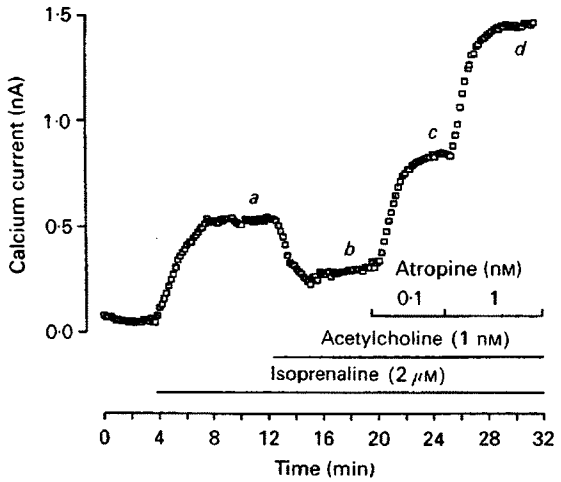

Figure 1 Atropine behaves as an inverse agonist on calcium currents (I_{Ca}) in frog ventricular cells (reproduced with permission from Hanf *et al.*, 1993).

It may be concluded from the above examples, that even in 'classical' organ bath or cell preparations inverse agonism may be readily demonstrated. This led us to examine older studies that show indirect evidence of some form of inverse agonism 'avant la lettre'.

Further indirect indications of inverse agonism

Within the concept of a (simplified) two-state receptor model (Figure 2), ligand efficacy may be redefined as the differential affinity of the ligand for the two conformational states (R, R*) with full agonists exhibiting higher affinity for the 'active' conformation (R*) and full inverse agonists for the 'inactive' conformation (R) (Monod *et al.*, 1965; Leff, 1995). This differential affinity may be detected in radioligand binding studies with the use of appropriate 'modulators' of the receptor state. For example, in several receptor systems it is known that guanylyl nucleotides (e.g. GTP in the μM–mM range) uncouple the G protein from the receptor leading to a 'low' affinity state of the receptor for agonists (Chang & Snyder, 1980; De Lean *et al.*, 1980; Stiles, 1988; Lohse *et al.*,1984). Thus, 'GTP-shifts' have been extensively used, in some GPCR fields, as a parameter to discriminate full from partial agonists due to the differential affinity of these ligands for the two receptor states (IJzerman *et al.*, 1996; Ehlert *et al.*, 1985).

However, extrapolation of this concept to antagonists that are able to discriminate between the free and G protein-bound form of the receptor may reflect, and even correlate with, the extent of inverse agonistic properties (negative intrinsic activity) of these antagonists. Although a regulatory role of GTP on antagonist binding, which is inverse to the role of GTP on agonist binding, has been previously suggested (Burgisser *et al.*, 1982; Ströher *et al.*, 1989) we should like to point out its significance and consequence that seems to have escaped attention somewhat.

Examples of guanylyl nucleotide enhancement of binding of several antagonists (as have been reported in literature) are shown in Table 3. Both affinity and B_{max} values of A_1 adenosine receptor antagonists [^{3}H]-XAC and [^{3}H]-DPCPX, on rat adipocyte and guinea-pig brain membranes, respectively (Ramkumar & Stiles, 1988; Ströher *et al.*, 1989), were increased significantly in the presence of GTP, indicating a preference of these antagonists for the uncoupled receptor state. A similar increase in binding was observed with two muscarinic cholinergic antagonists, [^{3}H]-QNB on frog heart membranes (Burgisser *et al.*, 1982) and [N-methyl-^{3}H]-scopolamine methyl chloride on rat heart membranes (Berrie *et al.*, 1979).

Accordingly, differential increase in binding of antagonists has also been observed in pertussis-toxin-mediated uncoupling

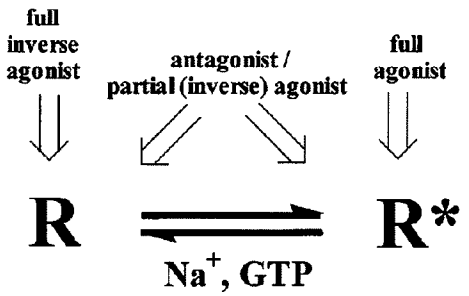

Figure 2 Simplified model representing the two conformational states of a receptor (inactive R, and active R*) and their differential affinities for ligands.

Table 3 Indirect indications of inverse agonism *in vitro*

Reference	*Assay system (tissue or cell membranes)*	*Receptor involved*	*Ligands (antagonists) used*	*Effect (shift)*
Ströher *et al.*, 1989	Guinea-pig brain	A_1Ado	[^{3}H]-DPCPX	GTP
Burgisser *et al.*, 1982	Frog heart	mACh	[^{3}H]-QNB	Gpp(NH)p
Berrie *et al.*, 1979	Rat heart	mACh	[N-methyl-^{3}H]-scopolamine	GTP
Ramkumar & Stiles, 1988	Rat adipocyte	A_1Ado	[^{3}H]-XAC	GTP
Costa & Herz, 1989	NG108-15	δ opioid	ICI-174,864	PTX
Appelmans *et al.*, 1986	Rat brain	δ opioid	ICI-174,864	Na^+
Nunnari *et al.*, 1987	Porcine brain	α_2-AR	[^{3}H]-yohimbine	Na^+
Neve *et al.*, 1980	Rat brain	Dopamine D_2	[^{125}I]-epidepride	Na^+
Pert & Snyder, 1974	Rat brain	Opioid	[^{3}H]-haloxone	Na^+
Horstman *et al.*, 1990	COSM6[1]	α_2-AR	[^{3}H]-yohimbine	Na^+
Gierschik *et al.*, 1989	HL60[2]	FP[3]	–	GTPase/Na^+
Szekeres & Traynor, 1997	NG108	δ opioid	–	GTPase/Na^+
Costa *et al.*, 1990	NG108-15	δ opioid	–	GTPase/Na^+

[1]Transfected with the wild type receptor. [2]Cell line derived from malignancy. [3]Formyl peptide.

of receptor and G protein; Costa & Herz (1989) were once more the first to show a leftward shift (increase in binding) upon toxin treatment in the competition curve of the δ opioid receptor antagonist ICI-174,864. The absence of any shift of antagonist MR2266 indicated a potential correlation of the negative intrinsic activity of ICI-174,864 with its greater affinity for the uncoupled form of the receptor.

Along these lines, interaction of sodium ions with several GPCRs is presumed to result in a stabilization of the low affinity (R) conformation of receptors (Green, 1984; Nunnari *et al.*, 1987). It is believed that this effect of Na^+ ions is linked to an aspartate residue in transmembrane helix II, conserved in virtually all GPCRs (Horstman, 1990). This effect of Na^+ ions has been shown to be correlated with intrinsic activity of ligands (Tsai & Lefkowitz, 1978), the largest Na^+-induced reduction in binding being observed for full agonists. However, although Na^+ ions in almost all cases inhibit strongly agonist binding, their effect on 'antagonist' binding has been variable, ranging from no effect to an increase in binding (Chang & Synder, 1980; Pert & Snyder, 1974; Nunnari *et al.*, 1987; Green, 1984). Although no explanation was given for this variability of effect among the different 'antagonists', it is now tempting to speculate that the inverse agonist behaviour of some may be responsible for the degree of 'Na^+-shift' they exhibit.

Hence, upon retrospective consideration, extrapolation of this concept may shed a new light on the observed effects of Na^+ ions. The increase in binding of some antagonists in the presence of Na^+ ions, such as [^{3}H]-naloxone to the μ opioid receptor (Pert & Snyder, 1974), ICI-174,864 to the δ opioid receptor (Appelmans *et al.*, 1986), [^{3}H]-yohimbine to the α_2-AR (Nunnari *et al.*, 1987) or [^{125}I]-epidepride to the D_2 receptor (Neve *et al.*, 1990), may be indicative of the inverse agonistic properties of these ligands. The fact that naloxone has been proposed to act as an inverse opiate agonist in a guinea-pig ileum preparation (Cruz *et al.*, 1996) is further support for this correlation.

Finally, one may note that apart from differential binding of ligands, the effect of Na^+ on the $R \rightleftarrows R^*$ equilibrium is also apparent in the reduction of basal levels of [^{35}S]-GTPγS binding (Szekeres & Traynor, 1997) or GTPase activity (Gierschik *et al.*, 1989; Costa *et al.*, 1990) in *in vitro* assays. This reduction is not only similar to the effect of inverse agonists themselves on these assays (decrease of basal receptor activity), but confirms the presence of constitutively active receptors in these assay systems. Thus, although the presence of Na^+ ions in radioligand binding assays may help to identify inverse agonists, their absence improves the ability of the [^{35}S]-GTPγS assay to expose inverse agonism.

Tonically active systems: a connection with constitutive receptor activity?

In physiology there are many examples of receptor systems tonically regulating a certain effect. This 'tone' has traditionally been attributed to the presence of the endogenous agonist of the receptor system involved that interacts at a continuous level with its receptors. Interestingly, under the new light shed by the concepts of the two-state receptor in equilibrium, it is tempting to set forth constitutive activity of the receptor system as the potential mechanism of receptor-mediated tone regulation, instead of -or in addition to- the role of endogenous agonists.

As an example, the cannabinoid receptor system tonically regulates thermal nociceptive thresholds in mice. SR141716A, characterized as an inverse agonist at CB_1 receptors (Landsman *et al.*, 1998; MacLennan *et al.*, 1998; Rinaldi-Carmona *et al.*, 1998), produces hyperalgesia in mice (in contrast to agonists in this system that produce analgesia) by inhibiting this tone (Richardson *et al.*, 1997). The same compound alone increased voltage-dependent Ca^{2+} currents in neurons microinjected with cloned CB_1 receptor RNA, reversing the tonic CB_1 receptor activity (Pan *et al.*, 1998). Again, for an 'antagonist' to elicit such an effect some receptors must be tonically active. Since care was taken to ensure the absence of endogenous agonists in this experimental set-up, the 'tone' could only be attributed to constitutive receptor activity and its reversal only to 'antagonists' with inverse agonist properties.

Another related example is the marked relaxation by oxytocin receptor antagonists F314 and F792 of spontaneous contractility of human myometrium *in vitro* (Kinsler *et al.*, 1996). It is interesting to postulate whether or not spontaneous uterine contractions during preterm labour are due to constitutive oxytocin receptor activity and whether, therefore, inverse agonists should be preferred over 'neutral' antagonists in treating such a condition. It should be mentioned in this respect that the levels of G_s vary dramatically in pregnancy, and hence the coupling with the oxytocin receptor. During gestation G_s α-subunit levels are substantially increased in human myometrium, whereas a significant downregulation of G_s is observed during parturition (Europe-Finner *et al.*, 1994).

Naturally occurring inverse agonists

The potential existence of naturally occurring or endogenous inverse agonists would be another line of evidence in favour of the physiological relevance of inverse agonism. Although not without dispute, agouti protein is considered an endogenous inverse agonist. This protein consists of 131 amino acids and is encoded by the *agouti* gene. Lu *et al.* (1994) were the first to report that agouti protein competitively antagonized melanocyte stimulating hormone (α-MSH) binding to its receptor, currently referred to as the melanocortin MC_1 receptor, with high affinity in the subnanomolar range. Siegrist *et al.* (1997) followed this observation and investigated the interactions between α-MSH, agouti protein, cyclic AMP elevating agents and phorbol ester on mouse B16 melanoma cells that endogenously express the MC_1 receptor. Cell proliferation, measured in one of the assays, was inhibited by α-MSH. Also agouti protein dose-dependently inhibited B16 cell growth, thus acting as an agonist. However, low concentrations of α-MSH counteracted the growth inhibition induced by agouti protein. Furthermore, melanin production and MC_1 receptor regulation were studied. Agouti was shown not only to decrease basal levels of melanin but also α-MSH-induced melanin production. In contrast to the cell proliferation assay, agouti protein had effects opposite to α-MSH, acting as an inverse agonist. However, the effects of α-MSH and agouti protein on receptor regulation were again comparable, i.e. both ligands downregulated the MC_1 receptor over a similar time course and to a similar extent. The authors provided two possible explanations for these differential effects. Either agouti protein could be interacting at a second site downstream in the MSH signalling pathway, implying a second putative agouti receptor, or agouti protein may be regarded as an inverse agonist on the MC_1 receptor. If this were the case agouti protein would interfere with a certain level of constitutive activation of the MC_1 receptor. However, constitutive activity of this receptor was not clearly shown in either study, preventing an explicit demonstration of inverse agonism. Therefore, the conclusion that agouti protein indeed is an inverse agonist on the MC_1 receptor cannot be drawn decisively at present.

Exendin-(9-39) is a truncated form of exendin-4, a peptide isolated from the venom of the lizard *Heloderma suspectum*. It acts as an inverse agonist on the murine glucagon-like peptide-1 (GLP-1) receptor (Serre *et al.*, 1998). Exendin-(9-39) dose-dependently decreased basal cyclic AMP levels in murine β-cells, which endogenously express the GLP-1 receptor. Neither endogenous GLP-1 nor preproglucagon mRNA could be demonstrated in these cells. Therefore exendin-(9-39) did not act as a 'neutral' antagonist preventing endogenously produced GLP-1 from binding to the GLP-1 receptor. Moreover the inverse agonistic effect of exendin-(9-39) was also observed one step downstream the signalling pathway. In mouse β-cells GLP-1 receptor activation results in the stimulation of glucose-induced secretion of insulin. Exedin-(9-39) inhibited this insulin secretion, corroborating the concept that inverse agonism has strong physiological relevance. Inhibition of glucose-induced insulin secretion by exendin-(9-39) acting *via* human GLP-1 receptors has not been shown yet. In conclusion, exendin-(9-39) is an inverse agonist from an animal species, although acting on a receptor of another species. This finding could stimulate the search for truly 'endogenous' peptides as inverse agonists.

The opposite 'combination' has also been described. Interferon-γ-inducible protein 10 (IP-10) is an endogenous ligand, acting as an inverse agonist on a viral receptor. Human IP-10 was shown to inhibit the basal activiy of a GPCR encoded within the genome of Kaposi's sarcoma-associated herpes virus/human herpes virus 8 (Geras-Raaka *et al.*, 1998). This GPCR shows homology with chemokine C-X-C receptors and is also referred to as ORF-74 (Rosenkilde *et al.*, 1999). C-X-C receptors are the natural target of IP-10 where activation of these receptors results in IP production. However, on ORF-74, which is constitutively active, IP-10 acts as an inverse agonist, decreasing basal IP levels. Human monokine induced by interferon-γ (Mig) is also a C-X-C receptor agonist, but lacks the ability to decrease basal signalling of ORF-74.

Rosenkilde *et al.* (1999), when analysing various other C-X-C receptor ligands, such as Growth-Related Oncogenes, Stromal cell-Derived Factor 1α (SDF-1α) and viral Macrophage Inflammatory Protein-II (vMIP-II), showed that besides IP-10, SDF-1α and vMIP-II behaved as inverse agonists on ORF-74. However vMIP-II, like ORF-74 itself, is also encoded by the human herpes virus 8 and acts as an antagonist on multiple human chemokine receptors. Thus, endogenous ligands (agouti or IP-10) have been identified as inverse agonists although not always acting on receptors present in the same organism (e.g. IP-10 for ORF-74 or exendin-(9-39) for GLP-1).

Implications for pharmacotherapy and drug design

Since the emergence of the concept of inverse agonism, (re)classification of existing and new compounds in an agonist-antagonist-inverse agonist continuum has become necessary.

Although it is hard to avoid reference to 'neutral antagonists' vs to inverse agonists, we are now becoming more aware that such a class of compounds does not really exist, or if so it is extremely limited. That is because an accurate definition of a neutral antagonist would be reserved for a ligand with exactly the same affinity for the active and inactive receptor conformation, something probably very rare. On the contrary, it is easier to speculate on 'partial inverse agonists' with various degrees of efficacy and variable affinity for these two receptor states. Thus, maybe we should realise that there is only one class of ligands after all, i.e. agonists, whether they are full, partial, partial inverse or full inverse. However, for convenience we will continue hereby to use the term 'antagonist' and /or 'neutral antagonist'.

It is now obvious that both mechanism of action and side-effect profile may largely vary for the two classes, antagonists and inverse agonists, especially in pathological conditions. Therefore it is imperative in drug therapy to clearly identify therapeutic agents that may act as either antagonists or inverse agonists. In some cases desired effects may be obtained by treatment with an antagonist, while in other situations an inverse agonist might be more effective. We will now discuss some observations that may have clinical relevance or strong implications for lead finding in drug discovery.

Effects of long-term treatment with inverse agonists

It has been shown for various receptor types that long-term treatment with inverse agonists is associated with upregulation of the receptor involved. The increase in receptor density may be associated with drug tolerance and withdrawal effects. For example, the marketed histamine 'antagonists' cimetidine, ranitidine and famotidine, which have been shown to be inverse agonists, upregulated rat histamine H_2 receptor numbers (Smit *et al.*, 1996). Theoretically, upregulation of the receptor involved could account for withdrawal effects and

deterioration of the disease upon cessation of the drug. In such a case, treatment with a neutral antagonist rather than with an inverse agonist should be considered a more rational choice of therapy. However, upregulation by cimetidine was less pronounced in studies with human H_2 receptors (150% over control cells versus 180% at rat H_2 receptors) (Alewijnse *et al.*, 1998). Moreover, a correlation between the level of upregulation of the receptor and withdrawal effects has not been found yet. To our knowledge, clinical relevance of histamine H_2 receptor upregulation has not been reported so far.

Upregulation of β-adrenoceptors upon antagonist treatment has been reported on many occasions. In one of the earlier studies the receptor density on membranes prepared from lymphocytes of healthy human subjects was determined after 8 days of drug treatment (Molinoff & Aarons, 1983). Propranolol increased β-AR density 140% over basal, while the agonists ephedrine and terbutaline decreased receptor density to approximately 50%. Pindolol, a so-called β-blocker which can express intrinsic sympathomimetic activity (hence, a partial agonist), also reduced receptor levels, but to a lesser extent. Abrupt discontinuation of propranolol treatment from patients with ischaemic heart disease is known to result in withdrawal effects such as increasingly severe and frequent anginal attacks, arrythmias and myocardial infarction. These side effects might be correlated with the increase in receptor number. Since pindolol decreased the β-AR density, compared to an increase of receptors by propranolol, it was suggested that discontinuation of long-term pindolol administration would not lead to outspoken withdrawal symptoms. However, the effect of drug treatment on receptor density appeared different in other studies with different experimental settings. For example, Hughes *et al.* (1988) reported downregulation of β-AR by propranolol in cultured lymphoma and muscle cells. Extrapolation of observations from such studies to the clinic is difficult and should be done with great care.

Upregulation of opiate receptors has also been reported. Quantitative autoradiography after chronic naloxone infusion, showed increased amounts of κ opioid receptors in specific regions in rat brain (Morris *et al.*, 1988). Furthermore, naloxone has been used to try and elucidate the mechanism of opiate tolerance/dependence and withdrawal (Cruz *et al.*, 1996). Its effects were assayed *in situ* in both morphine-treated and control guinea-pig ilea. In this study, naloxone counteracted morphine-induced neurodepression, but it also caused an (undesired) abstinence response. This withdrawal phenomenon, especially seen after bolus dosing, was interpreted as an abrupt change from the active (R*) to the inactive (R) receptor conformation. In this hypothesis tolerance to morphine is a reflection of the persistence of the active state of the receptor.

Long-term treatment with inverse agonists might have effects on other receptors as well. The G protein involved or receptors sharing the same signalling pathway as the target receptor could be affected. Bouaboula *et al.* (1999) described cross talk between different receptors *via* G_i proteins. SR144,528 not only acted as an inverse agonist on the cannabinoid CB_2 receptor (see above), it also prevented MAPK activation in response to insulin or lysophosphatidic acid by inhibiting Gi activity. Furthermore, Western blot analysis revealed G_i protein upregulation upon sustained treatment with SR144,528, which was reversed by washing and further exposure of the cells to the agonist CP-55940. To account for these observations, the existence of an inactive CB_2/G_i protein complex, in which the G_i protein is physically trapped and thus unavailable for other receptors, was suggested.

MacEwan & Milligan (1996), however, did not observe upregulation of G_s protein upon long-term treatment with inverse agonists for the β_2-AR, expressed in NG108-15 cells. Levels of other G protein subtypes (G_q and G_{11}) were also unaffected by sustained treatment with inverse agonists. Therefore, the regulatory properties of SR144528 on G protein levels might not be a common feature of all inverse agonists.

Recently, Berg *et al.* (1999) reported on the long-term treatment of the serotonin 5-HT_{2c} receptor. In CHO cells, expressing the 5-HT_{2c} receptor at a relatively low expression level ($\sim$250 fmol mg^{-1} protein), prolonged incubation with the inverse agonist SB206553 led to an increased responsiveness of the receptor to agonists (homologous sensitization). This was measured as an increase in maximal IP production induced by the agonist DOI. Interestingly, IP production was also enhanced by ATP, mediated *via* endogenous purinergic P_2 receptors (heterologous sensitization). These findings are quite comparable to the cross talk between receptors discussed by Bouaboula *et al.* (1999). In conclusion, long-term effects may be a sensitive measure of ligand properties.

Inverse agonists and somatic receptor mutations

Somatic receptor mutations leading to constitutively active receptors are a causal factor in certain diseases (Table 4). Inverse agonists may be beneficial here, since they would decrease the high basal activity induced by the mutation, while antagonists would have no effect. These mutations have been reviewed by Spiegel (1996), hence, we will discuss one example only.

The mutations H223R and T410P in the human receptor for parathyroid hormone (PTH) and parathyroid hormone-related peptide (PTHrP) have been found in patients with Jansen's metaphyseal chondrodysplasia. Both mutant receptors, expressed in COS-7 cells, caused an increase in basal cyclic AMP production, but to a different extent. Furthermore, they had apparent binding affinities for the natural ligands (PTH and PTHrP) that were approximately two times higher than those of the wild type receptor (Schipani *et al.*, 1996). Gardella *et al.* (1996) used these two mutant receptors to screen for peptide ligands as inverse agonists.

An interesting and somewhat related phenomenon is the RNA editing of receptors. This is a post-transcriptional modification of RNA transcripts due to the enzymatic conversion of adenosine to inosine. Niswender *et al.* (1999) demonstrated that as a result, the human $5HT_{2C}$ receptor exists in several isoforms in human brain. These isoforms all displayed less constitutive activity than the non-edited receptor upon expression in NIH3T3 fibroblasts and lower agonist potencies. This may imply that RNA editing is a physiological mechanism for fine-tuning synaptic signalling.

Inverse agonists and 'auto-receptor antibodies'

Inverse agonists may also play an important role in the treatment of autoimmune diseases. Mijares *et al.* (1996)

Table 4 Diseases associated with activating mutations (Spiegel, 1996)

Receptor	*Disease*
Rhodopsin	Retinitis pigmentosa, congenital night blindness
PTH-PTHrP	Jansen's metaphyseal chondrodysplasia
LH	Familial male-limited precocious puberty
TSH	Familial hyperthyroidism, toxic thyroid adenomas
Ca^{2+}	Autosomal dominant hypoparathyroidism

Table 5 Activating antibodies in autoimmune disease

Autoantibody	*Pathology*	*Reference*
β_1-AR	Idiopathic dilated cardiomyopathy	Magnusson *et al.*, 1990
β_2-AR	Chagas' disease	Guillet *et al.*, 1992
β_2-AR	Myasthenia gravis	Eng *et al.*, 1992
mACh M_2 receptor	Idiopathic dilated cardiomyopathy	Fu *et al.*, 1994
β_1-AR and β_2-AR	Ventricular arrythmias	Chiale *et al.*, 1995
mACh M_2 receptor	Chagas' disease	Elies *et al.*, 1996

analysed an antibody against a peptide corresponding to the second extracellular loop of the human β_2-AR. Monoclonal antibodies for this peptide were obtained from rabbits and their effects on guinea-pig cardiomyocytes were studied. A whole-cell patch clamp technique was used to assess the influx of Ca^{2+} ions by L-type calcium channels (I_{Ca}). Zinterol, a specific β_2-selective partial agonist, increased I_{Ca} 14% compared to the 100% increase in I_{Ca} induced by isoprenaline, a non-selective full agonist. Addition of the purified antipeptide antibodies to the cardiomyocytes increased I_{Ca} to a similar extent as zinterol. This effect appeared to be mediated by β-adrenoceptors and was β_2-selective. Addition of the neutral antagonist alprenolol did not decrease antibody-stimulated I_{Ca}, and on the contrary a significant increase was observed. ICI-118,551, an inverse agonist, blocked the effect of the antibodies completely, decreasing I_{Ca}. The results were interpreted in the light of the two-state receptor model based on the hypothesis that the antibodies recognized an epitope which is only presented at the active conformation of the receptor (R*). ICI-118,551 would shift the equilibrium towards the inactive conformation of the receptor (R), covering up the epitope recognized by the antibody. Consequently, the antibody is no longer able to bind and activate the receptor and therefore, an antibody-mediated change in I_{Ca} is not observed. Alprenolol, on the other hand, does not change the existing equilibrium between R and R* in this concept. Since some of the receptors are present in the R* conformation, the antibodies can still recognize the epitope on the receptor and elicit an increase of I_{Ca}.

It has been reported that autoantibodies against the second extracellular loop of certain GPCRs are involved in the pathology of human autoimmune diseases. Examples are listed in Table 5. These autoantibodies are believed to behave in a similar way compared to the anti-peptide antibodies described above. If the autoantibodies do activate the receptor, drug therapy with inverse agonists could be more effective than treatment with neutral antagonists. Moreover, conformation 'sensitive' antibodies can be used as a research tool to distinguish inverse agonists from neutral antagonists.

Inverse agonism as screening tool in drug discovery

Nowadays molecular genetic approaches have provided numerous orphan GPCRs with unknown function and/or ligand. Therefore robust functional assays need to be developed, preferably in high throughput screening formats. The concept of inverse agonism might provide new opportunities in such screening strategies. A functional assay in which a certain level of spontaneous activity is established could not only identify activating compounds (agonists) but also compounds with opposite effect (inverse agonists). In a classical screening assay, i.e. without constitutive activity, the latter ligands would not be detected at all. Screening strategies implementing inverse agonism may thus be more successful, significantly expanding 'hit' chances. Depending on the role of the orphan receptor, one of the classes can be further explored to discover and/or design new therapeutics. Furthermore, if inverse agonists are identified in such an assay, the medicinal chemist may use them as lead structures for further optimization and the discovery of e.g. more or less 'neutral' antagonists.

Epilogue

The physiological relevance of inverse agonism is not only becoming clearer, but is slowly taking a place and establishing a role in drug therapy. Further, it dictates a new field in drug research and especially in drug design, since it is obvious that new structure-activity-relationships for inverse agonists versus those for neutral antagonists must be sought for and established.

The authors acknowledge support from the EU BIOMED2 programme '*Inverse agonism. Implications for drug design*' (#BMH4-CT97-2152). We thank our colleagues in this programme Tommaso Costa, Susanna Cotecchia, Rob Leurs, Martin Lohse, Graeme Milligan, Nigel Shankley and Sir James Black. Their comments, discussions and critical reading of this manuscript were essential. A. Kourounakis also wishes to gratefully acknowledge financial support from the EU Biotechnology programme '*Novel concepts in the interaction between adenosine receptors and ligands: inverse agonists and allosteric enhancers*' (#BIO4-CT97-5138).

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(Received November 26, 1999
Revised February 18, 2000
Accepted February 18, 2000)

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Mechanisms of inverse agonism at G-protein-coupled receptors

Philip G. Strange

Many drugs with important therapeutic actions that had been assumed to be antagonists at G-protein-coupled receptors (GPCRs) have been shown to be inverse agonists. For both basic pharmacology and drug design it is important to understand the mechanisms whereby these drugs achieve their effects. It had been assumed that these drugs achieved their effects by stabilizing an inactive state of the receptor (R) at the expense of a partially activated state (R*). In this article, I consider this and other mechanisms that could explain inverse agonist actions, and conclude that more than one mechanism can apply to inverse agonism at GPCRs.

PRINCIPLES

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The concept of inverse agonism arose from the experimental observation that certain drugs were able to reduce the activity of receptor systems that were active in the absence of agonists. Given that agonists increase the activity of receptor systems this opposite or negative activity came to be known as inverse agonism. Inverse agonism was originally described for ionotropic receptors such as the $GABA_A$ receptor [1] but has subsequently been described extensively for G-protein-coupled receptors (GPCRs) [2–6]. In this article the mechanisms whereby inverse agonists, acting at GPCRs, might achieve their effects will be discussed.

The spectrum of efficacy

Although a few years ago it was thought that drugs acting at GPCRs could be divided into two classes, agonists and antagonists, it now seems that for most GPCRs the compounds acting at these receptors exhibit a spectrum of efficacy from inverse agonism through neutral antagonism to agonism. In the same way as agonists can exhibit a spectrum of activity from low-intrinsic-activity partial agonists to full agonists so can inverse agonists. Full and partial inverse agonists have been described in several systems (e.g. α_{1a}-, α_{1b}-, α_{2A}- and β_2-adrenoceptors, and 5-HT_{1A} receptors [7–10]), and very few drugs possess no efficacy, with only a few neutral antagonists having been recognized. In fact, many of the compounds that had previously been considered to be neutral antagonists are in fact inverse agonists. Some examples of drugs with important therapeutic actions

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Box 1. Mechanisms of inverse agonism based on the extended ternary complex model

For G-protein-coupled receptors (GPCRs) it has been widely assumed that inverse agonists suppress the agonist-independent activity of these receptors by stabilizing the receptor in an inactive state. This depends on receptor activation (receptor–G-protein coupling) occurring in the absence of the agonist, and the inverse agonist suppressing this activity in some way.

In terms of the extended ternary complex model [a] (Fig. I) inverse agonism can be achieved if $\alpha\beta<1$ (where α and β are allosteric constants governing the effect of the agonist on the R:R* and R*:R*G equilibria, R is the inactive form of the receptor, R* is the partially activated form of the receptor and G is the G protein). This means that the inverse agonist stabilizes the R form of the receptor at the expense of the R*G form of the receptor, thus suppressing agonist-independent activity. This general condition ($\alpha\beta<1$) can be achieved with various combinations of α and β but for clarity two models will be considered. First, inverse agonists could act by binding to the R state of the receptor in preference to the R* state (i.e. $\alpha<1$) (Model 1). Alternatively inverse agonists could bind to the uncoupled (R and R*) states of the receptor in preference to the coupled (R*G) state (i.e. $\beta<1$) (Model 2). These two models are considered in the main text.

A third possibility is that the inverse agonists do not redistribute the different states of the receptor. In this model (Model 3) binding of the inverse agonist switches the receptor to an inactive conformation, different from those shown above, that can exist in G-protein-coupled and uncoupled forms but is inactive. This model is partly a restatement of the cubic ternary complex model [b], which contains an inactive receptor conformation that can nevertheless couple to G proteins.

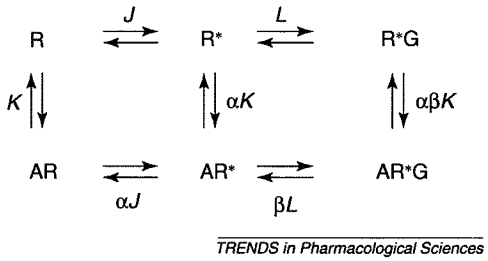

Fig. I. The extended ternary complex model. Abbreviations: A, agonist; G, G protein; *K*, the association constant for the binding of A to R; *J*, equilibrium constant governing the R:R* equilibrium; *L*, equilibrium constant governing the R*:R*G equilibrium; R, receptor; R*, partially activated receptor; α, allosteric constant governing the effect of the agonist on the R:R* equilibrium; β, allosteric constant governing the effect of the agonist on the R*:R*G equilibrium.

It should also be noted that no account has been taken here of possible effects of inverse agonists on the oligomerization state of the receptors [c].

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that have been shown to be inverse agonists include cimetidine (which acts on histamine H_2 receptors) [11], haloperidol (which acts on dopamine D2 receptors) [12], prazosin (which acts on α_1-adrenoceptors) [7], timolol (which acts on β_2-adrenoceptors) [9] and clozapine (which acts on D2 receptors and 5-HT_{2C} receptors) [12,13].

Physiological relevance of inverse agonism

Many drugs that had previously been considered to be antagonists have subsequently been shown to be inverse agonists in some assay systems. It is important to ask whether this matters in terms of drug effects in humans or animals. Many descriptions of inverse agonism rely on effects in recombinant systems but it is important to examine effects in native tissues. Acute administration of inverse agonists to native tissues might be expected to suppress the basal (agonist-independent) activity of a system. This depends on the native system exhibiting agonist-independent activation. Some examples of such effects in native systems have been described [5]. For example, histamine H_3 receptors in the brain have been shown to be constitutively active and suppression of this activation by inverse agonists has been described [14]. In this case, inverse agonists enhance histamine release from histamine-containing neurones by acting on H_3 autoreceptors providing a clear example of acute effects of inverse agonists in a native system.

Alternatively, inverse agonists might require chronic administration to exert their effects. There are many examples of the chronic administration of drugs that were considered to be antagonists leading to an upregulation of receptors [3]. This receptor upregulation could have important physiological implications. For example, prolonged treatment of patients with H_2 receptor inverse agonists such as cimetidine leads to the development of tolerance and an increased H_2 receptor sensitivity following withdrawal [11,15]. This could be related to upregulation of these receptors following drug treatment. It had been assumed in other systems that such effects were due to the prevention of the interaction of the agonist and receptor. However, it has been suggested that this upregulation is an expression of the inverse agonist property of the drug [3,6]. More examples of such effects of inverse agonists are required before the concept can be generalized.

Box 2. Use of constitutively active mutants of GPCRs to dissect the mechanism of action of inverse agonists

It has been widely assumed that inverse agonists reduce agonist-independent activation of G-protein-coupled receptors (GPCRs) by influencing the equilibrium between the inactive receptor (R) and the partially activated receptor (R*), stabilizing the R state of the receptor (Model 1, Box 1). If this is correct, it might be expected that a mutant receptor that favours the R* state of the receptor, a so-called constitutively active mutant, would exhibit a decreased affinity for an inverse agonist. Where this has been examined, the effects of such mutations on inverse agonist affinity have been very small in contrast to the effects on agonist affinity, which have been clear.

If we consider the extended ternary complex model as shown in Box 1 and consider conditions when receptor–G-protein coupling is prevented then the equilibria shown in Fig. I apply. The dissociation constant of a ligand that binds to the receptor is then given by the equation:

$$K_d = \left(\frac{1}{K}\right)\left(\frac{1+J}{1+\alpha J}\right) \quad [I]$$

For many receptors J (equilibrium constant governing the R:R* equilibrium) is relatively low so that there is little formation of the R* state when the agonist binds in the absence of G-protein coupling. For a constitutively active mutant receptor [where only the J constant is changed by the mutation (J')] the dissociation constant K_d' is given by:

$$K_d' = \left(\frac{1}{K}\right)\left(\frac{1+J'}{1+\alpha J'}\right) \quad [II]$$

The effect of the mutation on the dissociation constant of the ligand expressed as the ratio of the dissociation constants is then:

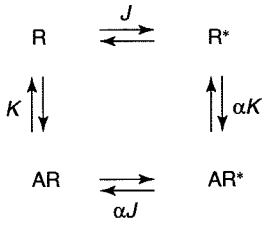

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Fig. I. Equilibria that exist in the extended ternary complex model when receptor–G-protein coupling is prevented. Abbreviations: A, agonist; K, the association constant for the binding of A to R; J, equilibrium constant governing the R:R* equilibrium; R, receptor; R*, partially activated receptor; α, allosteric constant governing the effect of the agonist on the R:R* equilibrium.

$$\frac{K_d'}{K_d} = \left(\frac{1+J'}{1+J}\right)\left(\frac{1+\alpha J}{1+\alpha J'}\right) \quad [III]$$

For an agonist, α (allosteric constant governing the effect of the agonist on the R:R* equilibrium) will be greater than one and so unless J and J' are both small there will be an effect of the mutation on the dissociation constant.

For an inverse agonist that stabilizes R, α is less than one and unless J' is greater than or equal to one there will be little effect of the mutation on the K_d of the inverse agonist.

It is possible to estimate the value of J in these mutants for the α_2- and β_2-adrenoceptors [a,b] based on the observation of a decrease in K_d of approximately twofold for some inverse agonists. If we assume that J is very low in the native receptor and that α is substantially less than one for an inverse agonist, the equation above reduces to:

$$\frac{K_d'}{K_d} = 1 + J' \quad [IV]$$

If K_d'/K_d is approximately two this gives a value of J' of one and the fraction of receptor in the R* state for the mutant receptor [$J'/(1+J')$] is ~50%. For the β_2-adrenoceptor, constitutive activation is in fact ~75% of the maximal agonist stimulation [b].

For other receptors, such mutants have been described and no change in the K_d':K_d ratio observed. This could be because the J' constant in the mutant is low but in some cases such mutant receptors exhibit high constitutive activation. For example, for the A293E mutant of the α_{1b}-adrenoceptor constitutive activation represents ~80% of the maximal agonist response [c]. We can estimate J' from the relation [activity = $J'/(1+J')$] as ~4, assuming that the increase in activation is entirely dependent on the change in J. Such a value of J' would predict a change in K_d that was measurable (approximately fivefold). This suggests that the mechanism of inverse agonism assumed might not hold.

One caveat about the use of these mutants should be noted. It is not entirely clear, although it is often assumed to be the case, that the mutants mimic the activated state of the receptor accurately or that the effects of the mutation are restricted to effects on J. This might require substantial additional characterization of the mutant to provide such verification.

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Mechanisms of inverse agonism

Thus, inverse agonists might exert their effects either acutely or chronically. Understanding the mechanism of the effects of inverse agonists is important because it could provide new ways to design drugs of defined intrinsic activity. During activation of ionotropic receptors, these receptors undergo a series of allosteric transitions between different conformational states [16] so that inverse agonists presumably stabilize certain states of the receptor with different functional activities.

For GPCRs, it has been assumed that inverse agonists act by switching the receptors to an inactive state [17]. Three mechanisms whereby this could be achieved will be considered (Box 1). Model 1, which states that the inverse agonist switches the R:R*(inactive receptor:partially activated receptor) equilibrium in favour of R, is the most widely discussed formulation of the actions of inverse agonists. Therefore, it is important to examine the evidence for this mechanism. It should be noted that in the discussion below the R:R* equilibrium refers to these states in the extended ternary complex model [17] and

not the alternative formulation of Leff [18].
I have chosen to use the extended ternary complex model here because it describes most of the phenomena under consideration. The cubic ternary complex model [19] is thermodynamically more complete but the extra complexity is not generally required here.

Stabilization of the R state of the receptor by inverse agonists at the expense of the R* state

One of the first tests of the proposal that inverse agonists stabilize the R state of the receptor at the expense of the R* receptor state was to use certain mutants of GPCRs that were thought to favour the R* state, the so-called constitutively active mutants [17]. If the R* state is favoured for these mutants and the inverse agonist binds preferentially to R over R*, it might be assumed that the affinity of the inverse agonist would be reduced by the mutation. However, this is a rather insensitive test for this mechanism of action [20] because there will be a large effect on the affinity of the inverse agonist only if the mutation causes the R* state to be strongly favoured. For many GPCRs the R* state is normally a very unfavourable state. Although these constitutively active mutations do increase the stability of R* and the affinity and potency of agonists is correspondingly increased, the extent of the increase in the stability of R* is such that the effects on the affinity of inverse agonists are not great (Box 2). Importantly, these mutations might not accurately reflect the activated state of the receptor [21].

Such mutations in α_2- and β_2-adrenoceptors have resulted in an approximate twofold decrease in inverse agonist affinity for these receptors [17,22]. These effects are consistent with Model 1 (Box 1) of inverse agonism, with ~50% of the receptor in the R* state in the mutant. By contrast, similar mutations in some receptors (e.g. α_{1b}-adrenoceptors) result in no change of inverse agonist affinity despite these receptors exhibiting substantial constitutive activity [7]. Thus, Model 1 of inverse agonism might not hold for these receptors. Some receptors exhibit only moderate constitutive activity and there is no effect of the mutation on inverse agonist affinity; in these cases it is not possible to identify the mechanism of inverse agonism from these data.

Thus, for some receptors, Model 1 of inverse agonist mechanism, whereby R is stabilized over R*, does hold. Further evidence for the inverse agonist switching the receptor to the R state comes from studies on phosphorylation of the β_2-adrenoceptor [20]. A very good correlation was reported between the ability of β-adrenoceptor kinase (βARK) to phosphorylate the agonist-occupied β_2-adrenoceptor for a range of agonists and the intrinsic activity of these agonists for stimulation of adenylyl cyclase [23]. In the absence of agonist very little phosphorylation of the native receptor was observed. By contrast, for a mutant β_2-adrenoceptor that is constitutively active and therefore might favour the R* conformation of the receptor, significant βARK-catalyzed phosphorylation was observed in the absence of agonist (i.e. there is constitutive phosphorylation) [20]. This constitutive phosphorylation was suppressed by 50% by the inverse agonist ICI118551, the affinity of which has also been shown to be reduced by the constitutively active mutation [20]. This work was performed in a reconstituted system in the absence of G protein so these data are consistent with the idea that the inverse agonist acts by binding to the R state of the receptor in preference to the R* state of the receptor.

In another approach to this problem, a purified native β_2-adrenoceptor bearing a fluorescent reporter group has been examined. The changes in the fluorescence of the reporter group were measured following the binding of ligands [24]. Changes in fluorescence of the reporter group were observed following binding of agonists and the extent of the change depended on agonist efficacy. Inverse agonists induced fluorescence changes opposite to those observed with agonists, providing evidence that the inverse agonists are not silent with respect to their effects on the native receptor. If the agonist-induced change in fluorescence corresponds to the R:R* interconversion, this suggests that the receptor normally lies partly in the R* state and the inverse agonist reverses this effect. The native β_2-adrenoceptor does exhibit some agonist-independent activation [17] in agreement with this hypothesis.

Thus, evidence in favour of Model 1 for the mechanism of inverse agonism exists for some receptors but not for others. The lack of effect of the constitutively active mutations on inverse agonist affinity for some receptors does not rule out Model 1 but it is nevertheless important to consider other models and other experimental approaches to inverse agonism.

Other mechanisms of inverse agonist action

*Stabilization of the R state over the R*G state*

One of the earliest descriptions of inverse agonism for GPCRs was for opiate receptors [25,26]. It was suggested that inverse agonists were switching the receptor between the RG and R states of the receptor. This depended on the sensitivity of the binding of inverse agonists to the effects of guanine nucleotides. Agonist affinity was decreased by GTP whereas inverse agonist affinity was increased and it was assumed that GTP was destabilizing the RG state. It was suggested that whereas agonists bound more tightly to the RG state of the receptor inverse agonists bound more tightly to the R state of the receptor. This mechanism was set out before the extended ternary complex model had been proposed but it is essentially Model 2 [i.e. the inverse agonist binds to the uncoupled states of the receptor in preference to the coupled states (Box 1)]. Therefore, it is important to examine evidence in favour of such mechanisms. Effects of guanine nucleotides on the binding of antagonists/inverse agonists have also been described for 5-HT_{1A} and 5-HT_{2C} receptors, muscarinic acetylcholine receptors in the heart (M_2 receptors), cannabinoid CB_1 receptors, adenosine A_1 receptors and dopamine D2 receptors [13,27–34].

Box 3. Use of agonist and inverse agonist binding to study mechanisms of inverse agonism

Ligand binding studies with agonists and inverse agonists can provide another tool to study the mechanism of action of inverse agonists. If we consider the extended ternary complex model as in Fig. I in Box 1, a neutral antagonist will label all of the species [R (inactive receptor), R* (partially activated receptor) and R*G (activated receptor bound to G protein)] with equal affinity. An agonist must stabilize R*G and it is generally assumed that this is achieved by the agonist exhibiting a higher affinity for the R*G species over the R and R* species ($\beta>\alpha>1$, where α and β are allosteric constants governing the effects of the agonist on the R:R* and R*:R*G equilibria, respectively). It should be noted that stabilization of R*G could be achieved in other ways; for example, a very strong stabilization of R* could lead to agonism even if R*G formation were less favourable ($\alpha>1$, $\beta<1$, but $\alpha\beta>1$). An inverse agonist could have a higher affinity for the R species over the R* and R*G species ($\alpha<1$, Model 1, Box 1) or it could have a higher affinity for the R and R* species over the R*G species ($\beta<1$, Model 2, Box 1).

These different states of the receptors can be probed using ligand binding. One example of this concerns the effects of guanine nucleotides on inverse agonist binding. If the inverse agonist binds preferentially to the uncoupled states of the receptor, binding might be increased in the presence of guanine nucleotides. This will occur only if there is substantial precoupling of receptor and G protein. Uncoupling of R and G by guanine nucleotide increases the amount of free receptor available to bind the inverse agonist with higher affinity. If R/G is greater than unity this might appear as an increase in B_{max} if the inverse agonist labels only the uncoupled form of the receptor with high affinity. Alternatively, the radiolabelled inverse agonist might label both the coupled and uncoupled forms of receptor under the conditions of the experiment. In this case the effects of guanine nucleotides might appear as a shift from two populations of sites to a single high-affinity population or might appear as an increase in inverse agonist affinity depending on the method of analysis used [a].

Alternatively, parallel radioligand binding competition assays with radiolabelled inverse agonist and agonist might probe the relative affinities of compounds for the different states. The radiolabelled agonist should label R*G with high affinity whereas the radiolabelled inverse agonist will label R with high affinity if Model 1 or 2 holds. If there is an excess of R over G and substantial precoupling of R and G then the two radiolabelled probes will label semi-independent pools of R*G and R, respectively. Hence, the affinities of compounds for the different states of the receptor can be examined. If a compound exhibits different affinities when competed against the two radiolabelled ligands this is some evidence that Model 1 or 2 holds.

It should be noted that if there is no precoupling of R and G, the inverse agonist will not show sensitivity to guanine nucleotides, even if Model 1 or 2 holds. The binding will be indistinguishable from that of a compound that has equal affinities for the different states of the receptor. Similarly, the compound will show similar affinities when tested versus a radiolabelled agonist or inverse agonist.

Some examples of receptors where inverse agonist binding shows different affinities when tested versus radiolabelled agonist or inverse agonist or where inverse agonist binding is sensitive to guanine nucleotides are given in the main text. However, it should be noted that even in these cases it is impossible to define the precise mechanism. Whether the inverse agonist stabilizes R over R* (Model 1) or R and R* over R*G (Model 2) these effects will be observed. More data are required to define the mechanism. For example, for the α_{2b}-adrenoceptor some compounds that have been shown to possess inverse agonist activity at the related α_{2a}-adrenoceptor subtype [b] do show different affinities when assayed versus radiolabelled agonist and inverse agonist [c]. There is some evidence for the α_{2a}-adrenoceptor that the R/R* mechanism holds based on the use of mutant receptors [b].

If, however, the binding of the inverse agonist does not show sensitivity to guanine nucleotides or does not show different affinities versus radiolabelled agonist and inverse agonist, in a system that is capable of this discrimination, then this rules out any mechanism where the inverse agonist redistributes the different states of the receptor (i.e. Model 3 must hold) and some examples of this are given in the main text.

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On the basis of the ability of spiperone to suppress agonist-independent [^{35}S]GTPγS binding, spiperone has been shown to be an inverse agonist at the 5-HT$_{1A}$ receptor [27]. The binding of [^{3}H]spiperone to this receptor is sensitive to the effects of guanine nucleotides so that spiperone exhibits a higher B_{max} in the presence of GTP [35]. Thus, this compound might stabilize the R state of the receptor at the expense of the R*G state (Model 2). Unfortunately, this observation cannot be used definitively to identify the mechanism. For example, if the inverse agonist were stabilizing R over R* (Model 1) the binding of the inverse agonist would also exhibit sensitivity to GTP [27]. The same will be true for other receptor systems where guanine nucleotide sensitivity of inverse agonist binding has been demonstrated. Model 1 or 2 could therefore apply and further work is required to differentiate the models. In the case of the 5-HT$_{1A}$ receptor, the two mechanisms of inverse agonism were probed [36] by making mutations of the 5-HT$_{1A}$ receptor that might favour R*. However, the mutations did not elicit constitutive activity in the same way as has been observed for other receptors and there were effects on the coupling of the receptor to different G proteins.

Radiolabelled agonist and inverse agonist binding

It is important to consider the use of another tool for probing the mechanism of inverse agonism that is related to the effects of guanine nucleotides described above. This concerns the evaluation of inverse agonist affinity in competition studies versus radiolabelled agonist binding and versus radiolabelled inverse

agonist binding (Box 3). If the extended ternary complex model holds for the system concerned, a radiolabelled agonist should label the R*G state with high affinity. A radiolabelled antagonist should label all states with similar affinity. A radiolabelled inverse agonist will label the uncoupled states (R or R*, depending on the model) with higher affinity and the R*G state with lower affinity. There might therefore be a difference in inverse agonist affinity when measured in competition assays versus radiolabelled agonist and inverse agonist. This will only be observed if there is precoupling of R and G in the system under study and an excess of R over G (Box 3). It should be noted, as above, that such a difference in affinity would be observed whether the inverse agonist bound preferentially to R over R*, or R or R* over R*G (Models 1 and 2). Further experiments are required to distinguish the two mechanisms (Box 3). It is only in the case where the inverse agonist does not show any affinity difference, in a system capable of such discrimination, that it is possible to conclude that the compound does not redistribute the affinity states (see below).

This test has been applied to the 5-HT_{1A} receptor [27], and spiperone shows a clear difference in affinity for the receptor when assessed in the presence of radiolabelled agonist compared with in the presence of radiolabelled inverse agonist. The observations with spiperone agree with the sensitivity of the binding of this ligand to guanine nucleotides. Methiothepin, however, does not show any difference in affinity when this is measured versus radiolabelled agonist and radiolabelled inverse agonist. Methiothepin, like spiperone, has been characterized as a full inverse agonist, and so these results tend to rule out mechanisms of inverse agonism via stabilization of R over R* and R over R*G for methiothepin (Models 1 and 2). Thus, more than one mechanism of inverse agonism can occur at one receptor when different compounds are considered.

For the D2 receptor expressed in recombinant cells, a range of inverse agonists has been tested in this way [37] and no differences have been found between affinities determined versus radiolabelled agonist or inverse agonist. There are, however, two reports of guanine-nucleotide-sensitive binding of spiperone, an inverse agonist, to D2 receptors in the pituitary [32,33] and one for domperidone in the brain [38]. These observations suggest that some inverse agonists at the D2 receptor are capable of discriminating the coupled and uncoupled states. This sensitivity to guanine nucleotides has not been observed in other experiments on D2 receptors in the pituitary [39], in the brain [40] or when expressed in recombinant cells (D. Roberts *et al.*, unpublished). One possible explanation for these differences would be that the D2 receptor undergoes different amounts of precoupling to G proteins in different systems, perhaps related to different populations of G proteins or accessory proteins. Inverse agonists are able to discriminate the coupled and uncoupled forms of the receptor in an appropriate context. Where there is precoupling the binding of inverse agonists will appear sensitive to guanine nucleotides whereas where there is no precoupling binding will appear guanine nucleotide insensitive. Further experimentation is required to resolve these issues.

As reported for methiothepin at the 5-HT_{1A} receptor, some inverse agonists show no difference in affinity to receptors when tested versus radiolabelled agonist or radiolabelled inverse agonist in a system that is capable of such a discrimination. This suggests that some mechanisms of inverse agonism do not involve a redistribution of the different receptor states by the inverse agonists (Models 1 and 2), but instead binding of the inverse agonist to the receptor switches the receptor to an inactive conformation that is unable to signal (Model 3). In this conformation, the receptor can still couple to G proteins but it cannot activate them. This concept of switching the receptor to an inactive state is similar to that proposed by Gether and Kobilka [41]. Similar mechanisms have been proposed for the muscarinic acetylcholine receptor based on functional studies [42]. The idea has been extended further for the cannabinoid receptors where it has been suggested that the inverse agonist promotes an 'active negative state' of the receptor that sequesters the G protein [30]. This is based on the ability of cannabinoid receptor inverse agonists to inhibit stimulation of mitogen-activated protein (MAP) kinase by insulin or the mas-7 stimulation of [^{35}S]GTPγS binding. These are both events that are independent of GPCRs but dependent on activated G protein. Thus, it was postulated that the inverse agonists were stabilizing an inactive state of the receptor–G-protein complex and sequestering G protein. It should be noted, however, that such behaviour is predicted by extending the model in Box 1 to provide the cubic ternary complex model [19], which explicitly allows for inactive receptor states that can couple to G proteins.

Sensitivity of inverse agonist binding to Na^+ ions

Another phenomenon that has been suggested to be related to inverse agonism is the apparent Na^+ ion sensitivity of the binding of certain inverse agonists to their receptors. For example, both the binding of yohimbine to the α_2-adrenoceptor [43] and the binding of the substituted benzamide drugs to D2 receptors [44] are of higher affinity in the presence of Na^+ ions. It has been suggested that this Na^+ ion sensitivity might be related to the mechanism of inverse agonism [5]. One possibility would be that these compounds bind better to R than R* (Model 1) and the effect of Na^+ ions is to stabilize R. However, this does not seem to provide a complete explanation because in mutant D2 receptors that favour R* the substituted benzamides exhibited unchanged Na^+ ion sensitivity [45]. Furthermore, for the D2 receptor there are compounds that show little sensitivity to Na^+ ions (e.g. haloperidol) and some (e.g. clozapine)

that bind better in the absence of Na^+ ions and these are all inverse agonists. Therefore, the Na^+ ion sensitivity of these D2 receptor ligands seems to be unrelated to their inverse agonism.

Concluding remarks

Inverse agonism can be achieved by more than one mechanism. For some compounds there is evidence that inverse agonism is achieved by the stabilization of the R state of the receptor over the R* state. For other compounds there is no redistribution of the different affinity states of the receptor but the inverse-agonist–receptor complex is inactive. This suggests that different inverse agonists stabilize a receptor in different conformations with different functional consequences.

Acknowledgements
I thank David Hall and Sam Hoare for their insightful comments on this paper.

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Chemical name

ICI118551: (±)-1-[2,3-(dihydro-7-methyl-1*H*-indeny-4yl)oxyl]-3-[(1-methylethyl)amino]-2-butanol

Inverse, protean, and ligand-selective agonism: matters of receptor conformation

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ABSTRACT **Concepts regarding the mechanisms by which drugs activate receptors to produce physiological response have progressed beyond considering the receptor as a simple on-off switch. Current evidence suggests that the idea that agonists produce only varying degrees of receptor activation is obsolete and must be reconciled with data to show that agonist efficacy has texture as well as magnitude. Thus, agonists can block system constitutive response (inverse agonists), behave as positive and inverse agonists on the same receptor (protean agonists), and differ in the stimulus pattern they produce in physiological systems (ligand-selective agonists). The molecular mechanism for this seemingly diverse array of activities is the same, namely, the selective microaffinity of ligands for different conformational states of the receptor. This paper reviews evidence for the existence of the various types of agonism and the potential therapeutic utility of different agonist types.—Kenakin, T. Inverse, protean, and ligand-selective agonism: matters of receptor conformation. *FASEB J.* 15, 598–611 (2001)**

Key Words: G-protein-coupled receptors · receptor activation · inverse agonism · receptor theory · constitutive receptor activity

NEW MOLECULAR TARGETS FOR DRUG DISCOVERY

THE PAST DECADE has brought an explosion of new information about G-protein-coupled receptors (GPCRs), their nature, and how they behave. With the sequencing of the human genome will come a plethora of new GPCR targets. This alliance of information is leading to a revolution in the drug discovery process. Similarly, there is a burgeoning number of chemical targets for therapeutic advantage; the list of drug targets for receptors has grown considerably (see **Fig. 1**). Before 1995, the major targets for drug development were full and partial agonists and antagonists. Since the principal mode of high-throughput screening has been radioligand binding, orthosteric ligands (those that sterically hinder the access of the radiolabel to the receptor binding site) primarily were discovered. Allosteric ligands (those that affect receptor function through binding to their own binding site separate from that of the endogenous ligand) were detected only if the allosteric interaction resulted in an alteration of the affinity of the receptor for the radiolabel. With the technological advances enabling high-throughput *functional* receptor screens should come an increase in the types of GPCR ligands in the new millennium. This will result in an increase in the number of allosteric ligands (modulators, agonists, enhancers) that modify receptor function without necessarily modifying steric access of the endogenous ligands to the receptor. Therefore, the changing mode of high-throughput screening can be predicted to lead to an increase in the texture of drug types for GPCRs. Another reason for the increasing number of drug targets is increased knowledge of GPCR behavior in cellular systems. This has led to the discovery of inverse agonism. This review will concentrate on a subset of these new chemical targets, namely, those that possess efficacy, either positive or negative, and these will be discussed in terms of their mechanisms of action and possible relevance to therapy of disease.

Drugs with 'efficacy'

Drugs can be thought of as having two properties with respect to biological systems: affinity for the receptor and intrinsic efficacy. A common usage of the word efficacy in clinical pharmacology is 'therapeutically useful activity'. Thus, a drug is considered 'efficacious' if it alleviates the symptoms of a disease in a patient. Within this context, even a competitive antagonist would have 'efficacy'. This review will discuss efficacy in terms of its formal definition in pharmacological receptor theory, that is, the property of a molecule that causes it to produce some observable physiological response. In terms of GPCRs, a useful working definition of receptor is the property of a molecule that causes the receptor to change its behavior toward the host system (1).

Three types of efficacious drugs will be discussed. Inverse agonists are an established drug class and possess what is termed 'negative efficacy'. Protean agonists are a theoretical class that produce receptor

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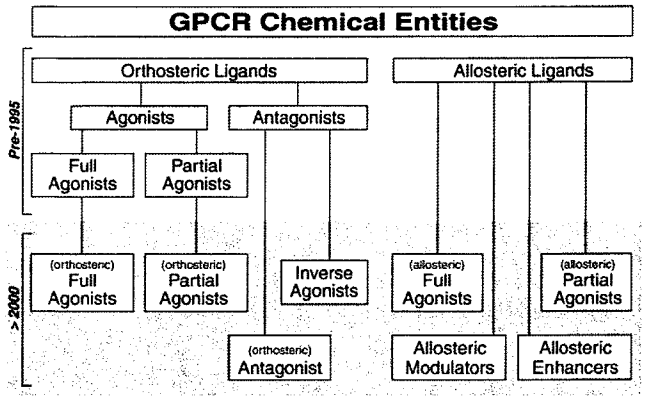

Figure 1. Chemical targets for GPCRs. Prior to 1995, the principal targets were full and partial agonists and antagonists. It is predicted that the types of ligands discovered will increase with increased screening technology. Full agonist: produces full receptor activation leading to production of the system maximal response. Partial agonist: produces submaximal receptor activation leading to production of submaximal system response and possible blockade of full agonist activation. Antagonist: produces no physiological response but rather blocks the response to endogenous or exogenous agonists. Inverse agonist: functions as an antagonist in nonconstitutively active systems, but has the added property of actively reducing receptor-mediated constitutive activity of GPCR systems (response not resulting from agonist activation but rather spontaneously emanating from the system itself). Allosteric agonist: functions as an agonist but activates the receptor through interaction at a site distinct from that of the endogenous agonist (usually a nonpeptide ligand for a peptide receptor). Allosteric modulator (antagonist): blocks receptor function but does not necessarily interfere with ligand receptor interaction (receptor occupancy). Allosteric enhancer: potentiates the effects of agonists on the receptor.

activation of lower magnitude than that emanating from spontaneous receptor constitutive activity. The predicted behavior for this class would be the observation of positive agonism in some GPCR systems and inverse agonism in others. Although this has been observed experimentally, an explanation of the effect in terms of receptor conformation is still theoretical. Finally, 'ligand-specific' agonism, which considers that some agonists have a different quality as well as quantity of efficacy for a given GPCR, will be considered. All of these classes will be discussed in terms of the evidence for their classification and their possible therapeutic relevance. As a preface to discussion of these drug entities, it is useful to discuss the dynamics of the GPCR systems with which they interact.

GPCR systems

G-protein-coupled receptors are allosteric proteins designed by nature to respond to small 'drug-like' molecules (i.e., neurotransmitters) to affect changes in large protein–protein interaction (receptors and G-proteins). The common currency of this translation of information is receptor protein conformation. It is essential to understand three particular properties of GPCR systems in order to understand how ligands can function as inverse, protean, and structure-specific agonists. The first is that, like all proteins, receptors can exist in various conformations. However, in the case of GPCRs, some of these conformations reveal sequences in their cytosolic loops, which can then activate G-proteins to initiate response. These conformations are referred to as the 'active state' (Ra) of the receptor; correspondingly, the conformation(s) that do not activate G-proteins are referred to as the 'inactive state' (Ri). In the simplest case, one single conformation of each will be assumed with the two conformations existing in an equilibrium defined by an 'allosteric constant' (denoted L and defined as [Ra]/[Ri]).

A second property of GPCR systems is that they are synoptic and interactive. Therefore, it is incorrect to describe GPCR function simply in terms of the receptor (two-state theory). Rather, the G-protein is an interactive and essential part of the system. The G-protein influences the receptor in ways that modify the behavior of the receptor and vice versa. Of particular relevance is the fact that a receptor can spontaneously interact with G-proteins in the absence of agonist ligands. Thus, if the affinity of Ri for a G-protein is denoted Kg (equilibrium association constant), the affinity of the active state Ra for the same protein is denoted βKg where β>1. Response emanates from the hydrolysis of GTP by the G-protein resulting from activation by Ra. From these elements the simplest version of a GPCR system can be constructed:

$$\mathrm{Ri} \overset{L}{=} \mathrm{Ra} + \mathrm{G} \overset{\beta Kg}{=} \mathrm{RaG} \qquad (1)$$

It can be seen that such a system defines the possibility of constitutive activity whereby a response can be produced by the GPCR system in the absence of an agonist. The system can be made to produce a response through stoichiometry of the reactants, namely, Ri and G. Thus, the constitutive activity (as defined by elevated levels of [RaG]) can be increased by raising the receptor concentration:

$$\text{Constitutive Activity} = \frac{\beta L[\mathrm{Ri}]/K_G}{1 + \beta L[\mathrm{Ri}]/K_G} \qquad (2)$$

where K_G is the equilibrium dissociation constant of the receptor/G-protein complex (K_G=1/Kg), or by increasing the concentration of G-protein:

$$\text{Constitutive Activity} = \frac{\beta L[G]/K_G}{1 + L(1 + \beta[G]/K_G)} \qquad (3)$$

Another way in which constitutive activity can be produced is through alteration of L, the allosteric constant. Under normal circumstances, L is a unique molecular constant for a given receptor (i.e., the energy barrier to formation of spontaneous active states for some receptors is lower than it is for others), but experimental methods such as the removal of sodium ions (2, 3) or point mutation (4–10) can affect L and make receptors more constitutively active.

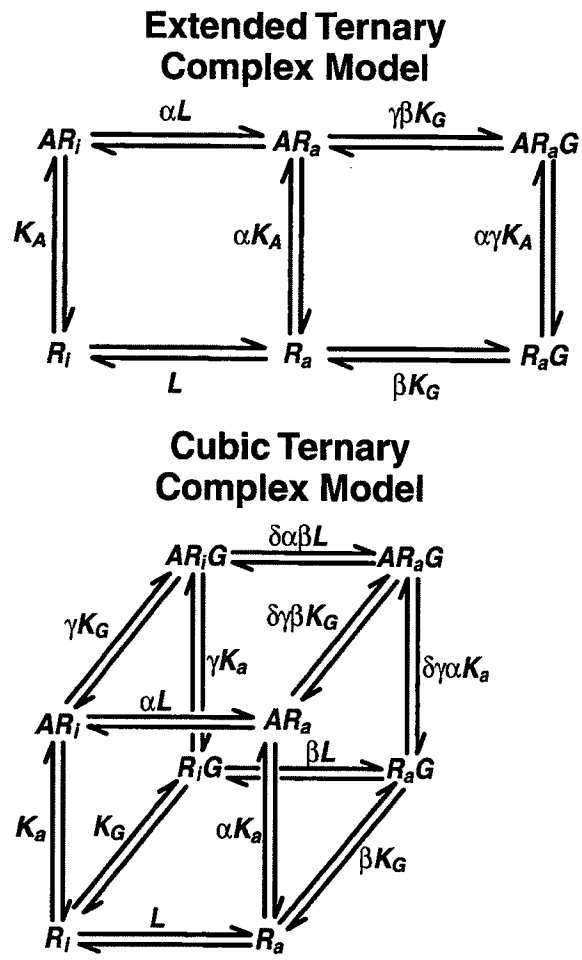

Figure 2. Two models for GPCR systems. The extended ternary complex (ETC) model (5) assumes that only the active-state receptor (Ra) can interact with the G-protein either spontaneously (to form RaG) or through ligand binding (to form ARaG). The association constants are K (ligand to receptor) and βK_g (receptor to G-protein). L is the allosteric constant and α, γ the modifiers of affinity once the receptor is active or ligand bound, respectively. The cubic ternary complex is very similar except it allows the inactive-state receptor Ri to interact with the G-protein as well (13–15).

The simple model for GPCR systems can be completed by adding the interaction of ligand (designated [A]) to the system to produce a corresponding array of species ARi, ARa, and ARaG. When the ligand-bound ensemble is added to the scheme shown in Equation 1 (see also **Fig. 2A**), the extended ternary complex model (ETC model) for GPCR systems results (5). A more thermodynamically complete version of the system allows the inactive receptor Ri to interact with the G-protein. In terms of thermodynamic modeling, this must be allowed to occur (11). However, the existence of an inactive ternary complex comprising ARiG is largely theoretical. Some examples of this complex can be found for some receptors (see ref 12 for a review); however, the thermodynamically complete model for GPCR systems, termed the cubic ternary complex model (CTC model; 13–15), requires a greater number of microaffinity constants than the ETC model and generally is more complex (see Fig. 2*B*). The ETC model can be regarded as a subset of the CTC model and adequate for GPCR systems for which the interaction of Ri with G-protein is thought to be minimal. For the purposes of this review, both models yield similar predictions for GPCR behavior with some minor exceptions.

The third relevant property of GPCR systems is an extension of the first: the production of multiple *active* receptor states (that go on to produce response through interaction with G-proteins). The minimal requirement for a GPCR model is that one receptor active state be formed. Thus, in principle, agonists can induce response by causing enrichment of that single receptor active state. Under these circumstances, efficacy would then be a matter of the quantity of the active state produced by the agonist. However, there is no theoretical constraint on the number of receptor active states. Even though the ETC and CTC model have both been referred to as 'two-state' models, this is a misnomer in that there is the capability within both to be multi-state models. The two-state aspect of these models refers only to the unliganded species Ri and Ra. In principle, the microaffinity constant of the liganded receptor could be specific for the ligand (through the values α and γ for the ETC model and α, γ, and δ for the CTC model; see Fig. 2), i.e., the affinity of the ligand-bound receptor for G-protein (ARaG) could be different from the unbound form (RaG). Under these circumstances, both the ETC and CTC models can accommodate an infinite number of receptor active states for agonism.

It is clear that proteins, including GPCRs, can adopt numerous conformations according to thermal energy (16, 17). What is not clear is what proportion of these conformations are capable of activating G-proteins, i.e., how many are receptor active states? Amino acid sequences have been identified in the intracellular loops of GPCRs that, when exposed to G-proteins, activate them (18–20). In fact, small oligopeptide isolated sequences have been found to activate G-proteins on their own (21, 22). With this model in mind, it would suggest that the inactive form of the receptor prevents access of G-proteins to these sequences, thereby precluding receptor activation of G-proteins. The corollary to this is that any disruption of the tertiary structure of the receptor could expose these activating amino acid sequences to initiate G-protein activation. On theoretical grounds, it might be expected that there could be numerous tertiary conformations of the receptor capable of exposing these intracellular sequences, i.e., there could be numerous active state conformations of the receptor. Mutation studies support this idea. For example, the substitution of 20 amino acids in position 293 of the α_{1A}-adrenoreceptor produces a constitutively active receptor—essentially 20 different active state similar forms of the α_{1A}-adrenoreceptor (4). The production of constitutive activity (whereby the receptor spontaneously adopts an active state and produces

G-protein activation) through such mutations for receptors indicates that disruption of receptor tertiary conformation can expose activating sequences to G-proteins (10, 23, 24). A general message from these studies is the possibility of the existence of numerous active state conformations of GPCR able to initiate physiological response. The apparent ligand-specific production of receptor conformations that interact differently toward other membrane proteins (including G-proteins), to be discussed later in the context of ligand-specific agonist efficacy, further suggest the existence of multiple receptor active states for GPCRs.

The previous discussion has described essentially three characteristic behaviors of GPCR systems: the capability to exist in multiple states, the ability of these states to spontaneously interact with other membrane proteins, and the possible existence of multiple states capable of inducing physiological response. To explore the interaction of ligands with such systems, it is useful first to discuss the mechanism by which ligands can influence receptor/G-protein ensembles.

The influence of ligands on GPCR systems

The relative quantities of various protein species existing in equilibria with each other are governed by the equilibrium dissociation constants that define their ratio. Thus, the allosteric constant is defined as [Ra]/[Ri]. The nature of L is controlled by the molecular nature of the receptor; thus, for any quantity of Ri there will be a quantity of Ra governed by the magnitude of L. However, this can be changed if external forces perturb the quantity of either one of the species. For example, if a ligand binds selectively to the Ra species to form ARa, then the quantity of free Ra is depleted and the magnitude of L will dictate that more Ra must be formed at the expense of existing Ri (see Scheme 1).

This can be shown mathematically within the constraints of either the ETC or CTC model (Fig. 2). For example, the concentration of response producing species (RaG and ARaG) in the presence of a ligand A in terms of the ETC model is given by Kenakin et al. (12):

$$\rho = \frac{\beta L[G]/K_G(1 + \alpha\gamma[A]/K_A)}{[A]/K_A(1 + \alpha L(1 + \gamma\beta[G]/K_G)) + 1 + L(1 + \beta[G]/K_G)} \quad (4)$$

In the absence of agonist ([A]=0)

$$\rho_0 = \frac{\beta L[G]/K_G}{1 + L(1 + \beta[G]/K_G)} \quad (5)$$

In the presence and absence of a maximal concentration of ligand (saturating the receptors; [A] $\rightarrow \infty$)

$$\rho_\infty = \frac{\alpha\gamma\beta L[G]/K_G}{1 + \alpha L(1 + \gamma\beta[G]/K_G)} \quad (6)$$

The ratio of response producing species in the presence and absence of ligand is given by:

$$\frac{\rho_\infty}{\rho_0} = \frac{\alpha\gamma(1 + L(1 + \beta[G]/K_G))}{(1 + \alpha L(1 + \gamma\beta[G]/K_G))} \quad (7)$$

As depicted in Fig. 2, α and γ reflect modifiers of the affinity constant of the receptor for the G-protein when the receptor is activated and occupied by ligand, respectively. For example, a value of $\alpha > 1$ indicates a greater affinity of the ligand for the active-state receptor Ra. It can be seen from Equation 7 that only one condition will enable a ligand to bind to the GPCR species in the system and *not* cause a redistribution of receptor species. That is if $\alpha = \gamma = 1$ (the presence of the ligand on the receptor does not in any way affect the affinity of the receptor for G-proteins, i.e., the ligand has no efficacy). If α or $\gamma \neq 1$, then the ratio of active-state species will change in the presence of A: when A is added to the system, the concentrations of the various species will redistribute. Therefore, the selective affinity of ligands for various receptor conformations will change the overall distribution of species in GPCR receptor ensembles and thus, either induce or inhibit response. This is the basic mechanism of ligand efficacy and the basis for the molecular nature of inverse, protean, and ligand-selective agonism.

INVERSE AGONISTS

Inverse agonists were discovered only after the tools with which they could be detected were created, namely, constitutively active GPCR systems. Whereas ligands that depress the basal benzodiazepine receptor (a non-GPCR) activity had been studied a decade before (25, 26), true GPCR constitutive activity was first quantified in recombinant receptor systems where experimental conditions could be manipulated to produce constitutive activity. As noted, the ability to spontaneously produce an active receptor conformation (the conformation that activates G-proteins) is defined by the allosteric constant, a receptor-specific constant defining the energy barriers to the production of tertiary conformations. Thus, in natural systems with defined receptor/G-protein stoichiometry, the amount of spontaneously formed active-state receptor species may not be sufficient to demonstrate visible constitutive receptor activity. This constraint was eliminated by the introduction of recombinant GPCR systems, which could experimentally manipulate the relative stoichiometry of receptors and G-proteins.

A classic study by Costa and Herz (2) of NG108–15 cells recombinantly expressing opioid receptors was instrumental in defining constitutive GPCR activity and inverse agonism. Costa and Herz (2) produced a system that responded to the classic opioid agonist (i.e., [D-Ala^2), D-Leu]enkephalin), but also had an elevated basal response and demonstrated a depression of basal activity with the peptide ICI 174864 ([N,N'-diallyl-Tyr^1,Aib^{2,3}]Leu^5-enkephalin). In this constitutively active GPCR system, ICI 174864 depressed the ligand-independent elevated basal responses and was thus

defined as an inverse agonist. The simplest mechanism by which inverse agonism could occur is the selective affinity of the ligand for the inactive state of the receptor. Thus, as the ligand binds selectively to Ri, the receptor species in the system will redistribute. If the system has RaG present (constitutive activity), then this species will be depleted as more receptor transforms into ligand-bound Ri; the result will be a decrease in constitutive activity.

Inverse agonism is a fairly newly discovered phenomenon for GPCR systems. The effect was initially met with some skepticism since it required the reclassification of established antagonists as inverse agonists. Also, in some systems the trace presence of endogenous agonists leads to an apparent constitutive activity, which could then be depressed by simple competitive antagonists, i.e., inverse agonism could be an artifact in some systems. However, the lack of depression of basal responses to some antagonists (i.e., neutral antagonists) and the use of such neutral antagonists to block the effects of inverse agonists clearly indicate that the phenomenon is real. For example, Costa and Herz (2) used the neutral antagonist MR 2266 to block the effects of the positive agonist DADLE and the inverse agonist ICI 174864, and showed that the potency for the inhibition of both effects was the same.

After the initial discovery, there was a period when there was a paucity of data available to judge the prevalence of inverse agonists in pharmacology. However, with time has come an increasing number of reports describing previously classified antagonists as inverse agonists. This rise coincided with the increased availability of recombinant and constitutively active GPCR systems, a prerequisite for the observation of inverse agonism. Thus, now that more laboratories have eyes to see inverse agonism, the more it has been seen.

It is still premature to judge the prevalence of inverse agonism in chemical space. In theoretical terms, there is reason to believe that all ligands should not possess efficacy. As described above, for a ligand *not* to cause redistribution of GPCR species it must recognize at least two receptor conformational species as being identical: Ra and Ri. In a constitutively active system, this is increased to three species by the presence of RaG. As shown in Equation 7, the ligand-specific constants α and γ must be unity in terms of the ETC model (and α, γ, δ in the CTC model) for redistribution not to occur (i.e., for a ligand to have no efficacy). The question then is: How often, in thermodynamic terms, is this likely to occur? Although some studies appear to support the prediction that most antagonists are inverse agonists (i.e., of 23 α_1-adrenoreceptor antagonists of varying structure, all were inverse agonists) (27), there are clear examples of neutral antagonists in the literature. The degree of inverse agonism observed depends on the relative affinity of the inverse agonist for the various receptor species and the degree of constitutive activity in the system. Thus, ligands that only slightly differentiate receptor conformations will essentially appear to be neutral antagonists, especially in systems with low levels of constitutive activity.

Although the existence of inverse agonists has been substantiated in experimental systems, the therapeutic relevance of this drug class is as yet unknown. It also is not clear whether negative efficacy would be a desirable or undesirable property to have in an antagonist molecule. In the absence of constitutive receptor activity, an inverse agonist behaves exactly as a simple competitive antagonist. However, if there is constitutive activity present in the therapeutic system, then, unlike a simple competitive antagonist, an inverse agonist will depress the resulting elevated basal response. There are physiological scenarios where this may or may not be advantageous.

Adverse effects of inverse agonists

Inverse agonism has been associated with receptor up-regulation leading to tolerance to chronic antagonism. For example, in treating an ulcer, tolerance to some histamine H2 receptor antagonists has been observed (28–30). It has been postulated that chronic treatment with histamine antagonists results in increased levels of membrane histamine receptors (31). The ligands shown to cause increases in histamine H2 receptor density—cimetidine and ranitidine—are inverse agonists but there is no concomitant increase in receptor density observed with the neutral antagonist burimamide (32). In that membrane receptor populations are not static, but rather are a series of steady states resulting from receptor synthesis, transport to the surface, internalization, and degradation, any ligand that perturbs receptor states theoretically can affect the steady-state level of the receptor density. For example, activation by agonists increases phosphorylation of many receptors and subsequent internalization (33–35). It has been shown that spontaneous formation of receptor active states (constitutive activity) leads to eventual internalization of receptor as well (33, 36). Possessing equal affinity for both the inactive and active receptor states, a neutral agonist would not alter flow of receptor to and from the membrane surface. However, an inverse agonist could halt the spontaneous cycle of receptor synthesis, transport, internalization, and degradation at the membrane by selectively stabilizing the inactive state of the receptor. If this state is more resistant to phosphorylation and subsequent internalization, then receptor degradation would be slowed in the face of unaltered receptor synthesis. The extent of change of steady-state membrane receptors would be a function of the rates of the various processes synthesizing, transporting, and internalizing them (37), but under appropriate conditions elevations of receptor could occur leading to increased agonist response. This, in turn, would result in a decrease in the effectiveness of the antagonist. Thus, in this scenario, inverse agonism would be an undesirable property (38). Receptor up-regulation by inverse agonists has been shown to occur with inverse agonists for histamine H2

receptors (32), β_2-adrenoreceptors (39), and α_1-adrenoreceptors (40). In addition to changes in receptor density, inverse agonists have also been found to alter levels of G-protein. Thus, up-regulation of levels of $G_{q/11\alpha}$ through 5-HT_{2C} receptors (41) and $G_{s\alpha}$ through β_2-adrenoreceptors (42) has been obtained with inverse agonists for the respective receptors. Presumably the changes in receptor stimulation of these pathways leads to secondary effects on G-proteins.

Therapeutic application of inverse agonists

The extent to which inverse agonism could be a therapeutic advantage depends on the role of constitutive GPCR activity in pathology. One potential therapeutic area where this might have relevance is cancer. It has been shown that chronic elevation of second messengers in cells produced by constitutive G-protein activity can lead to cell transformation (43–45). For example, receptors such as the α_1-adrenoreceptor have been shown to be agonist-independent proto-oncogenes (46). Constitutive GPCR activity leading to chronic elevation of cell metabolism may also have a role in promoting the growth of tumors. There are examples of high levels of expression of specific GPCRs in tumor cells; it has been shown that endogenous ligands for these receptors are present at high levels in the tumor cells (self-regulation) and that they have proliferative properties. There also is evidence to show that inhibition of the cellular effects of these ligands can inhibit tumor growth.

One such receptor is vasoactive intestinal peptide receptor (VIP). Receptors for VIP are found in high density in a number of tumors (47–55); see **Table 1**. In fact, these high levels of VIP receptors can be used to image tumors through binding of ^{123}I-VIP (55) and ^{123}I-labeled octreotide (VIP ligand; 57, 58) binding.

The relevance of high levels of VIP GPCR activity on tumors relates to the fact that this peptide promotes growth and proliferation of normal and malignant cells (59–63). Inhibition of VIP function in these cells leads to a decrease in cancer growth (64, 65); see **Fig. 3**. The relevant question for inverse agonism is, to what extent can the VIP-mediated proliferation be attributed to constitutive VIP GPCR activity? Many of these tumors have high levels of VIP, and it has been suggested that VIP secretion from these tumors regulates VIP receptor expression on the same cells (66). Certainly the high levels of VIP receptor present on the tumor cell membrane would make them extremely sensitive to low levels of released VIP. However, the sheer magnitude of the receptor expression suggests that constitutive receptor activity may also play a role in the pathology.

TABLE 1. *VIP receptors in tumors and normal cells*

Cells	B_{max} (sites/cell)[a]	Multiple of platelet cell density
Platelets	$2.1 \pm 0.3 \times 10^3$	1
A431[b]	$1.6 \pm 0.3 \times 10^6$ (high-affinity sites)	800×
	$9.7 \pm 0.4 \times 10^6$ (low-affinity sites)	4620×
COLO 320[c]	$1.9 \pm 0.4 \times 10^8$ (high-affinity sites)	90,500×
	$7.3 \pm 0.8 \times 10^6$ (low-affinity sites)	347,600×
HT29[c]	$1.2 \pm 0.5 \times 10^8$ (high-affinity sites)	5700×
	$6.9 \pm 0.9 \times 10^6$ (low-affinity sites)	32,850×
PANC1[d]	$2.1 \pm 0.4 \times 10^8$ (high-affinity sites)	100,000×
	$6.9 \pm 0.8 \times 10^6$ (low-affinity sites)	32,850×

[a] Binding of ^{125}I-labeled VIP. [b] Epidermoid mammary carcinoma. [c] Adrenocarcinoma. [d] Pancreatic epitheloid carcinoma. From ref 56.

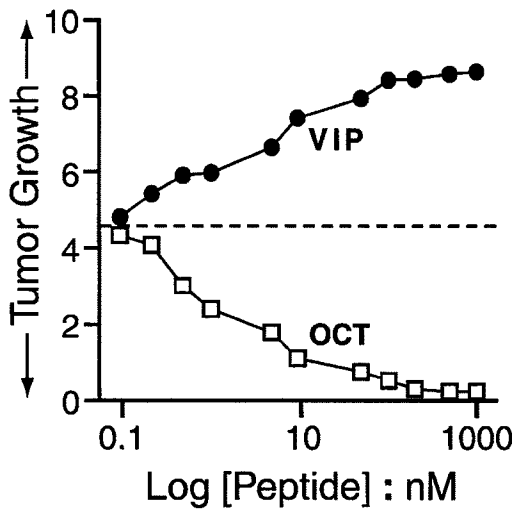

Figure 3. Effects of VIP and peptide fragment octreotide on tumor growth as measured by ^{3}H-thymidine-incorporation (cpm × 10^3). Abscissae are logarithms of molar concentration of vasoactive intestinal peptide (VIP) or octreotide. Redrawn from ref 56 with permission.

There are differences in the proclivity with which different GPCRs spontaneously produce an active-state receptor (with corresponding constitutive activity). Some receptors have a low-energy barrier for the formation of Ra (i.e., human calcitonin, chemokine CCR5, neuropeptide Y types 2 and 4) whereas others, such as NPY1, do not readily produce constitutive activity (67); the difference lies in the magnitude of the allosteric constant, L. However, since the definition of L is the ratio of Ra to Ri (L=[Ra]/[Ri]), then irrespective of the magnitude of L, a 1000- to 10,000-fold increase in the number of receptors will lead to a corresponding 1000- to 10,000-fold increase in the number of spontaneously existing active-state receptors. Thus, the magnitude of L for VIP would need to be exceedingly small to prevent such high levels of receptor from producing constitutive activity.

Another peptide of interest in cancer is bombesin. Bombesin, gastrin-releasing peptide, and VIP are related in that VIP may induce the release of bombesin/GRP in small cell lung cancer (65, 68). Bombesin-like peptides are potent mitogens, and a role has been proposed for them in oncogenesis and/or proliferation of malignant cells (69). Bombesin/gastrin-releasing peptides are found in high levels in small cell lung

carcinomas, suggesting that these could be autocrine factors for cancer growth (70–72). As with VIP, blockade of bombesin activity through monoclonal antibodies attenuates cancer growth (69).

Inverse agonists would both block the effects of humoral activation of these receptors on cancer cells (i.e., secreted VIP, bombesin) and constitutive activity in the tumor due to either receptor over-expression and/or mutation. Whereas the effect would be cytostatic rather than a cytocidal (tumor death would not be achieved), a reduction in tumor cell metabolic activity could be a useful adjunct to chemotherapy.

Certain disease states may be treated effectively *only* with inverse agonists. These are instances where the pathological entity is a constitutively active GPCR, which produces physiological response in the absence of endogenous agonists. For example, certain pathological mutations lead to constitutively active GPCRs, which in turn result in diseases such as retinitis pigmentosa and hyperthyroidism (see review by Spiegel, ref 73). Constitutively active GPCRs may also be important in autoimmune diseases (see review by de Ligt et al., ref 74). Viral infection also can lead to constitutively active GPCR pathology. For example, infection with Kaposi's sarcoma-associated herpes virus leads to expression of a constitutive chemokine receptor, which in turn elevates IP3 to lead to cell proliferation and continued viral replication (75, 76).

In general, it still is not clear to what extent GPCR constitutive activity plays a role in pathology. However, it is known that receptors and enzymes levels change in conditions of trauma (hypoxia, ischemia, physical damage), disease (inflammation, viral or bacterial infection), or development (78, 79). Although, in general, solid examples of constitutive receptor activity playing a role in disease are sparse, with the classification of clinically used inverse agonists, the relationship between negative efficacy and therapeutic utility should become clearer. Along with clarification of the role of constitutively active GPCRs in pathophysiology will come a measure of the value of inverse agonists in therapy.

PROTEAN AGONISTS

A unique reversal of drug activity, based on the notion that some agonists may produce an active receptor conformation of lower efficacy than the spontaneously formed one has been predicted on theoretical grounds (80, 81). These kinds of ligands were given the name protean agonists after Proteus, the Greek god who could change shape and appearance at will. In this case, the reversal from positive to negative agonism is protean. If a given agonist produces a receptor active state that is less efficacious (to be denoted [Ra′]) than the spontaneously formed one (denoted [Ra]), then in systems that are quiescent (no constitutive activity), the ligand would produce excitation by virtue of changing the predominant Ri into Ra′. However, if the system were constitutively active (significant amount of Ra), then the ligand would reduce the activity by changing Ra to Ra′. Therefore, in quiescent systems the ligand would be a positive agonist and in constitutively active systems it would be an inverse agonist. Presently it is not clear what therapeutic relevance such an agonist would have except perhaps to set the level of stimulation of a given system to a constant level. Thus, if pathology produced constitutive activity to create an overstimulation of the system or if the endogenous stimulus to the system were to be diminished by pathology, then a protean agonist would be useful if the maximal effect of the agonist was appropriate.

On the other hand, there is a considerable theoretical interest in protean ligands since they can act as a looking glass into agonist-specific receptor active states. Thus, the observation of protean agonism would be presumptive evidence that the ligand in question produces a receptor active state of lower intrinsic efficacy than the naturally occurring constitutively active state. It is worth considering the experimental conditions under which such protean agonism would be observed.

The starting point is to have a ligand that produces a positive agonist response in a quiescent (nonconstitutively active) receptor system. It might be supposed that the agonism should be partial (in keeping with a less efficacious ligand-bound active state). However, saturation of system stimulus-response mechanisms might allow low efficacy agonists to produce the full system response; therefore, partial agonism may not be a prerequisite. The next step is to observe the effect of ligand in a system where the receptor is made to spontaneously form the natural active state. For example, **Fig. 4*A*** shows the effect of increasing the magnitude of the allosteric constant L (as might be produced by removal of sodium ions) in a hypothetical GPCR system. The ligand is a theoretical drug that promotes the formation of the natural active state (α=100) but forms a ligand bound species that has a lower affinity for the G-protein than the natural active state (ARa has a lower affinity for G than does Ra; γ=0.01). Calculations with the CTC model show that in the quiescent system (L=0.01), the ligand is a positive agonist. Changing L from 0.01 to 0.3 elevates the basal response of the system and causes the ligand to demonstrate inverse agonism. Another way to produce constitutive activity is by increasing the amount of G-protein available to interact with the receptor (Equation 3). Under these circumstances, a similar ligand (γ=0.03) will demonstrate protean agonism as well (Fig. 4*B*). Another condition that may yield protean agonism is when the receptor reactivity to the G-protein changes. For example, Fig. 4*C* shows that if the affinity of both Ri and Ra is reduced for the G-protein (K_G increases), as might be produced by desensitization, an inversion of agonism for the same ligand would be observed. Note how in this case the basal activity is not altered.

There have been experimentally observed instances of protean agonism for β_2-adrenoreceptor ligands. For example, dichloroisoproterenol (DCI) is a positive par-

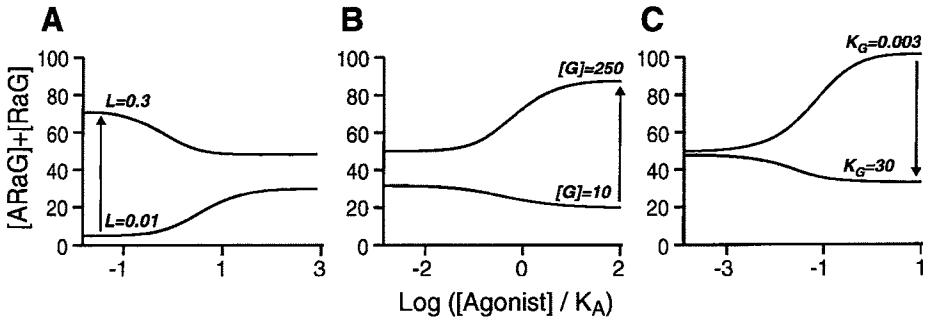

Figure 4. Three theoretical conditions that promote protean agonism. Simulation made with cubic ternary complex model (Fig. 2*B*) for a ligand with α = 100, γ = 0.01, δ = 0.1. [R] = 100. *A)* [G] = 100, K_G = 30, β = 10. The system ranges from quiescent (L=0.01) to constitutively active (L=0.3). *B)* Ligand with α = 100, γ = 0.03; L = 0.1; [G] increased from a value of 10 to 250. *C)* Ligand with α = 100, γ = 0.03, L = 0.1; receptor 'desensitized'; [G] = 500, interaction of receptor and G-protein efficient (K_G=0.003) to inefficient (K_G=30).

tial agonist for β_2-adrenoreceptors transfected into sf9 cells. Upon desensitization of the system through prolonged treatment with the full agonist isoproterenol (as depicted in the simulation Fig. 4*C*), DCI produces inverse agonism (82). **Figure 5** shows the effects of three β_2-adrenoreceptor ligands on transfected sf9 whole cells; DCI, labetalol, and pindolol all produce increases in cyclic AMP (positive agonism). However, when membranes were made from the same cells, the system became constitutively active (due to removal of GTP) and, under these circumstances, these same ligands produced depression of basal cyclic AMP levels (inverse agonism) (83). It is not clear to what extent low efficacy receptor conformations are responsible for the experimentally observed protean agonism. However, observation of the phenomenon is suggestive of selective receptor states and this may be a useful tool for discovery of ligand-specific receptor active-states.

LIGAND-SPECIFIC RECEPTOR ACTIVE STATES

Numerous lines of experimental evidence indicate that all agonists do not produce the same active state of

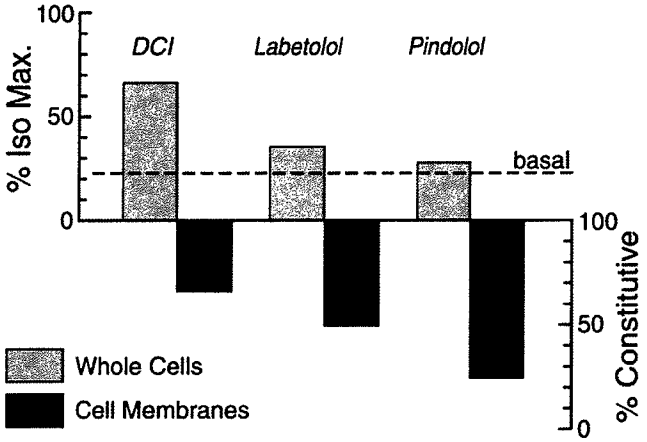

Figure 5. Experimentally observed protean agonism. Sf9 cells transfected with β_2-adrenoreceptor. Gray bars represent whole cells (not constitutively active due to presence of intracellular GTP); ligands produce stimulation of cyclic AMP. Membranes from same cells are constitutively active; the same ligands produce inverse agonism (filled bars). Data from ref 83 with permission; figure from ref 1 with permission.

GPCRs. One of the most compelling findings is the reversal of relative potency of agonists for receptors that activate more than one stimulus-response element. For example, the human 5-HT_{2C} receptor is coupled to two separate response pathways in CHO cells: phospholipase C-mediated inositol phosphate accumulation (IP accumulation) and phospholipase A2-mediated arachidonic acid release (AA release). The agonist (±)-1-(2,5-dimethoxy-4-iodophenyl)-2-aminopropane (DOI) produces a higher maximal stimulation than the 5-HT agonist quipazine for arachidonic acid release (77). Since maximal response is dependent only on efficacy, this indicates that DOI has a greater efficacy than quipazine for AA release. In contrast, the efficacies of the two agonists are reversed for the IP accumulation where quipazine has the greater efficacy. This cannot be explained by a uniform active-state receptor interacting with the two pathways identically for the two agonists, but rather it suggests that the active state formed by DOI (arachidonic release-preferring) is different from that produced by quipazine (IP accumulation-preferring). Similar reversals of efficacy have been reported for PACAP (pituitary adenylate cyclase-activating polypeptide) receptors (84), dopamine D_2 receptors (85) and *Drosophila* tyramine receptors (86). A striking reversal of relative potency of substance P analogs on neurokinin-1 receptors has been reported (87). Thus, whereas substance P is 2.1× more potent than the analog [P_3^Emet(O_2) (11)SP for producing cyclic AMP through NK-1 receptor activation, it is 0.11× *less* potent than the analog for producing phosphoinositol hydrolysis through activation of the same receptor. Whereas no reversals in relative efficacy for agonists was found in a study of CB1 cannabinoid receptors (known to activate both Gs and Gi protein), marked discontinuities in the activity of agonists were observed indicating that some agonists produced conformations that favored one of the two G-proteins while others did not (88).

Differential activation of G-proteins by receptors (referred to as stimulus trafficking; refs 89–91) cannot be accommodated by a mechanism whereby one single receptor active state produced by all agonists interacts with G-proteins. Although differential stimulus pathway activation can occur through strength of signal type of mechanism (i.e., a highly efficacious agonist may activate two pathways whereas a weaker agonist may acti-

vate only the more sensitive one), reversal of relative activity cannot be explained in this manner. Rather, the two G-proteins involved must see different conformations. It would be expected that different conformations of the receptor would have differential activation reactivities to different G-proteins since it is known that different areas of the cytosolic loops on receptors activate different G-proteins (92, 93). It would not be expected that different tertiary conformations of the receptor would expose these different G-protein-activating sequences in an identical manner.

Stimulus trafficking can be detected in specially designed recombinant GPCR systems. Referred to as stimulus-biased assay systems (94), these are hosts with identical cellular backgrounds except for the enrichment of a single Gα subunit. For example, human calcitonin receptors are pleiotropic with respect to the G-proteins with which they can interact (Gs, Gq, Gi; ref 95). Transfection of human calcitonin receptors (type 2, denoted hCTR2) into wild-type HEK 293 cells and HEK cells stably transfected with enriched populations of Gα subunits show striking differences in *relative* agonist potencies. **Figure 6*A*** shows that not only does the relative potency of eight calcitonin agonists on hCTR2, transfected in wild-type cells, and HEK cells stably enriched with Gαs subunit change, but so does their rank order of potency. Figure 6*B, C* shows dose-response curves to rat amylin and porcine calcitonin in wild-type cells and Gαs-enriched cells, respectively. It can be seen that the relative potency of the agonists changes from 4.6 to 84 with Gαs-enrichment, a finding that cannot be accommodated by the assumption that both agonists produce the same receptor active state (94). Rather ,it suggests that porcine calcitonin produces a conformation more conducive to using Gs than does amylin.

Other experimental approaches have furnished data to indicate differential G-protein activation by different agonists produced by agonist-specific receptor conformations. For example, the kinetics of adenylate cyclase activity in the presence of limiting GTP concentrations indicates a differential rate of heterotrimer dissociation for different $\beta_2$adrenoreceptor agonists (96). Similarly, whereas the efficacy of β_2-adrenoreceptor agonists for promoting GTP hydrolysis correlates well for the efficacy of the agonists for stimulating adenylate cyclase, the same is not true for the hydrolysis of inosine triphosphate. The differences in the ability of different agonists to hydrolyze GTP vs. ITP suggest that different receptor active states are produced (97).

There are still other lines of evidence to suggest that agonists produce ligand-specific receptor conformations. Selective mutations of dopamine D_2 receptors caused selective abolition of receptor/G-protein activation by dopamine but not other dopamine agonists. This suggests that these agonists produce different receptor conformations interacting with G-protein (98). Studies of the receptor desensitizing effects of different agonists also indicate the production of ligand-specific receptor conformations. For example, it

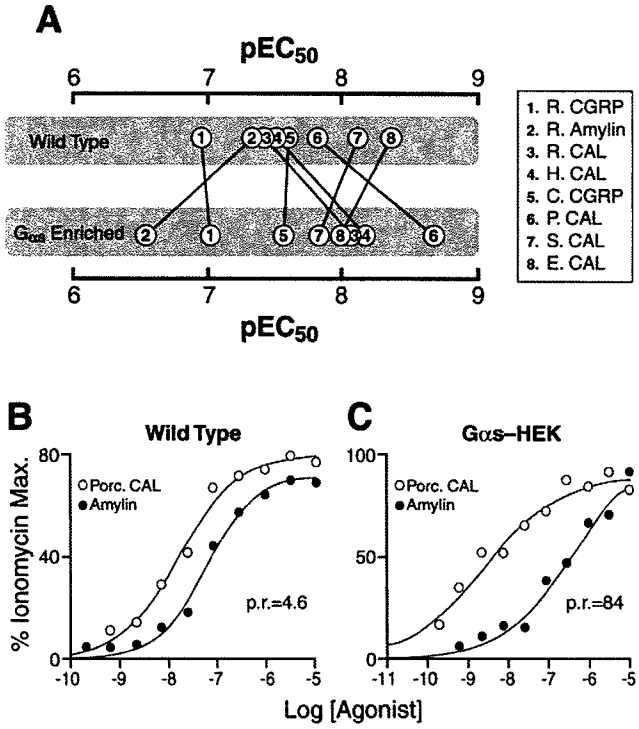

Figure 6. Relative potency of calcitonin receptor agonists in wild-type and stimulus-biased GPCR systems. *A)* pEC_{50} (−log of the molar concentration producing half maximal stimulation) for calcium mobilization (n=3) for 8 agonists (code shown in box: CAL=calcitonin, R=rat, H=human, E=eel, P=porcine, C=chicken, S=salmon) on calcitonin receptors (hCTR2) transfected into wild-type HEK293 cells (top bar) and HEK293 cells stably enriched with Gαs subunit (bottom bar). Change in pEC_{50} shown for each agonist by lines joining the points. *B)* Dose-response curves (calcium mobilization as measured by fluorescence) to porcine calcitonin (open circles) and rat amylin (filled circles) in wild-type HEK293 cells. The relative potency of calcitonin to amylin is 4.6. *C)* Relative potency of the same agonists as shown in panel *B* in Gαs-enriched stimulus-biased host cells. The relative potency is now 84. Data for panel *A* previously unpublished by Chris Watson, GlaxoWellcome Research, panels *B* and *C* from ref 1 with permission.

would be expected that the relative propensity of agonists to induce desensitization would parallel their relative efficacies. This was shown to be generally true for μ opioid receptor agonists, with the notable exception of methadone and L-α-acetyl methadone. These latter agonists produced disproportionate desensitization and receptor phosphorylation, suggesting different receptor conformational changes (99). Similarly, methadone and buprenorphine have been shown to demonstrate different desensitizing properties from morphine on μ opioid receptors (100). In other studies of recovery from desensitization, it has been shown that agonists appear to produce different conformations. Thus, whereas the recovery from prolonged activation of 5-HT_3 receptor with partial agonists is mono-exponential, it is sigmoidal (indicating 3 steps and 4 states) with full agonists (101).

The effects of agonists on receptor internalization also have furnished interesting data regarding ligand-

specific receptor conformation. Here it can clearly be shown that the simple strength of receptor stimulation can be differentiated from the ability of ligands to induce receptor internalization. For example, the cholecystokinin (CCK) receptor antagonist D-Tyr-Gly-[(Nle^{28,31},D-Trp^{30})cholecystokinin-26–32]-phenethyl ester does not produce receptor stimulation but rather blocks CCK responses. This antagonist also produces profound receptor internalization (102). Similarly, whereas enkephalins and morphine produce stimulation of δ and μ opioid receptors, enkephalins induce rapid receptor internalization whereas morphine does not (103). These data indicate that the conformations that lead to response are not necessarily the same as those that induce receptor internalization. It also suggests that different agonists produce receptor conformations with differential propensity to internalize.

In conclusion, diverse experimental approaches have provided evidence that ligands can stabilize different receptor conformations. Some of these conformations relate to receptor signaling, whereas others may relate to receptor sensitivity to endogenous agonist or presence on the cell membrane. The challenge is to exploit this behavior for therapeutic advantage.

Ligand-selective conformations and therapeutic utility: the quality of efficacy

Historically, receptors have been thought of operationally in terms of 'on-off' switches. In this context, efficacy was considered to be the 'on' position and the only gradation available in this scheme was degree of strength. With the possibility of agonist-selective activation of receptors and the definition of efficacy as a change in the behavior of receptors to their hosts comes the capacity to control the 'quality' of efficacy as well.

In terms of signaling, a common quest in drug discovery is to obtain ligands with a subset of activity for a given endogenous ligand receptor system. Historically, the method for doing this was through discovery of receptor subtypes. Thus, whereas epinephrine has a plethora of metabolic activities in the body mediated by β-adrenoreceptors, selective agonist stimulation of only the β_2-adrenoreceptor subtype provides useful therapy for asthma. Stimulation of the receptor subtype reduces the spectrum of metabolic responses produced by the general receptor family. If it is accepted that different receptor conformations most likely reveal different portions of the intracellular cytosolic loops of GPCRs, then ligand selective receptor conformations can lead to further selective directing of activation to G-proteins (trafficking of receptor stimulus). Such trafficking has been shown in natural and recombinant systems. For receptors that produce pleiotropic activation of multiple G-proteins, this would limit the signaling pathway activated by the particular ligand and thus confer further selectivity to the agonist (see **Fig. 7**).

It is not obvious how knowledge of ligand selective efficacy would be applied to drug discovery. However, it could be useful to classify agonists on the basis of stimulus-response coupling as knowledge for retrospective analysis. Currently, agonists are all assumed uniformly to stimulate receptors and differ only on a spectrum of strength of signal. Separating agonists, in terms of the stimulus-pathways that they preferentially activate, may offer insights into preferred profiles of agonism as compounds are progressed from screening assays into therapeutically oriented secondary assays (104).

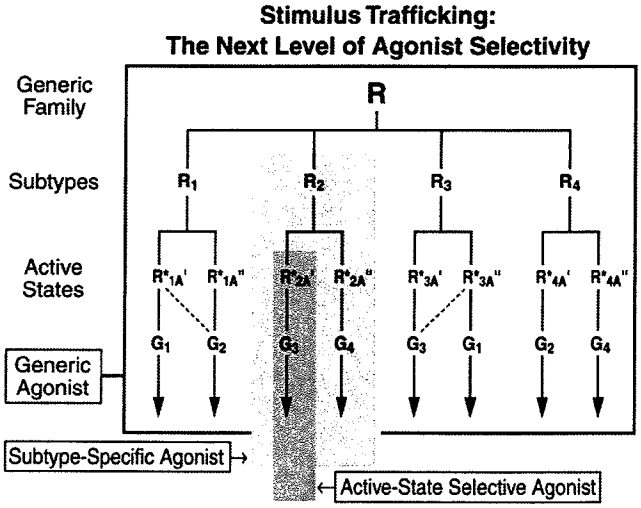

Figure 7. Schematic representation of the relative selection of stimulus pathways for receptors as a generic family is divided into subtypes and then each subtype is allowed to produce different active conformations that preferentially interact with different G-proteins; stimulus pathways are restricted. From ref 104 with permission.

There are other realms of ligand-selective receptor conformation selection that may have therapeutic utility. For example, ligands that selectively induce receptor internalization may have great utility in the prevention of HIV-1 infection through chemokine receptor fusion. Ligands that cause internalization of CXCR4 (105, 106) or CCR5 (107, 108) have been shown to protect against HIV-1 infection *in vitro*. The selective removal of chemokine receptor from the cell surface could be superior to blocking chemokine receptor interaction with HIV viral coat proteins because it would circumvent possible rapid emergence of resistant HIV variants through therapeutic pressure and mutation (109–111).

There are other realms where differential conformations leading to differences in receptor disposition could be useful therapeutically. For example, ligand-selective bias in the production of receptor desensitization could be beneficial in treatment of tolerance (99, 100). Similarly, receptor dimerization may be implicated in numerous areas including HIV-1 infection (112, 113) and the function of cannabinoid receptors (114), $GABA_B$ receptors (115–118), adenosine A1 receptors (119), δ-opioid receptors (120), β_2-adrenoreceptors (121), and calcium-sensing receptors (122–124). Ligands that induce selective conformations

affecting dimerization may produce unique effects not necessarily associated with direct receptor signaling.

Finally, it is becoming evident that GPCRs can associate with other membrane proteins to change their affinities to ligands and reactivities toward G-proteins. For example, receptor activity-modifying proteins can change the phenotype of calcitonin gene-related peptide, adrenomedullin receptors, and calcitonin receptors (125–129). Similarly, GPCRs are known to interact with other accessory proteins such as PDZ domain-containing proteins. Thus, β_2-adrenoreceptors interact with Na^+/H^+-exchanger regulatory factor (130) and 5-HT_{2C} receptors with MUPP1 (a multi-PDZ domain protein with no currently known function (131). Again, as with desensitization, dimerization, and internalization, these receptor functions could, in theory, be regulated differentially by different ligand-induced receptor conformations to change receptor function. This could lead to another dimension in control of the quality of ligand efficacy.

CONCLUSIONS

This review describes three apparently separate phenomena—inverse agonism, protean agonism, and different types of positive agonism—in terms of a single mechanism of action, namely, the interaction of different receptor conformations (some spontaneously formed and some ligand directed) with G-proteins. It can be seen that such a system has a vastly increased range of adjustment over one in which a single activated receptor interacts with G-proteins on a scale of strength of signal. In this scheme, the stoichiometries of cellular components can adjust GPCR system set points and sensitivities; ligands theoretically can bias such systems in a multitude of ways. The challenge for the next millennium in drug discovery and receptor pharmacology will be to exploit ligand bias in these complex systems for therapeutic advantage. FJ

I wish to thank Mrs. Donna McGhee for expert preparation of this manuscript.

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